PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/12, 15/64, C12Q 1/68, C07K 14/435 | A1

(11) International Publication Number: WO 96/26272

(43) International Publication Date: 29 August 1996 (29.08.96)

(21) International Application Number: PCT/US96/02424

(22) International Filing Date: 23 February 1996 (23.02.96)

(30) Priority Data:

08/394,152	24 February 1995 (24.02.95)	US
08/466,381	2 June 1995 (02.06.95)	US
08/470,735	2 June 1995 (02.06.95)	US

(60) Parent Applications or Grants
(63) Related by Continuation
US 08/394,152 (CIP)
Filed on 24 February 1995 (24.02.95)
US 08/466,381 (CIP)
Filed on 2 June 1995 (02.06.95)
US 08/470,735 (CIP)
Filed on 2 June 1995 (02.06.95)

(71) Applicant (*for all designated States except US*): SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH [US/US]; 1275 York Avenue, New York, NY 10021 (US).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): ISRAELI, Ron, S. [US/US]; 528 Liberty Avenue, Staten Island, NY 10305 (US). HESTON, Warren, D., W. [US/US]; Apartment 18B, 400 East 85th Street, New York, NY 10028 (US). FAIR, William, R. [US/US]; Apartment 3501, 400 East 70th Street, New York, NY 10021 (US).

(74) Agent: WHITE, John, P.; Cooper & Dunham L.L.P., 1185 Avenue of the Americas, New York, NY 10036 (US).

(81) Designated States: AU, CA, JP, MX, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: PROSTATE-SPECIFIC MEMBRANE ANTIGEN AND USES THEREOF

(57) Abstract

This invention provides an isolated mammalian nucleic acid molecule encoding an alternatively spliced prostate-specific membrane (PSM') antigen. This invention provides an isolated nucleic acid molecule encoding a prostate-specific membrane antigen promoter. This invention provides a method of detecting hematogenous micrometastic tumor cells of a subject, and determining prostate cancer progression in a subject.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

PROSTATE-SPECIFIC MEMBRANE ANTIGEN AND USES THEREOF

This application is a continuation-in-part of United States Application Serial Nos. 08/466,381 and 08/470,735, both filed June 2, 1995, which are continuations of U.S. Serial No. 08/394,152, filed February 24, 1995, the contents of which are hereby incorporated by reference.

This invention disclosed herein was made in part with Government support under NIH Grants No. DK47650 and CA58192, CA-39203, CA-29502, CA-08748-29 from the Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

Throughout this application various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of each set of Examples in the Experimental Details section.

Prostate cancer is among the most significant medical problems in the United States, as the disease is now the most common malignancy diagnosed in American males. In 1992 there were over 132,000 new cases of prostate cancer detected with over 36,000 deaths attributable to the disease, representing a 17.3% increase over 4 years (2). Five year survival rates for patients with prostate cancer range from 88% for those with localized disease to 29% for those with metastatic disease. The

rapid increase in the number of cases appears to result in part from an increase in disease awareness as well as the widespread use of clinical markers such as the secreted proteins prostate-specific antigen (PSA) and prostatic acid phosphatase (PAP) (37).

The prostate gland is a site of significant pathology affected by conditions such as benign growth (BPH), neoplasia (prostatic cancer) and infection (prostatitis). Prostate cancer represents the second leading cause of death from cancer in man (1). However prostatic cancer is the leading site for cancer development in men. The difference between these two facts relates to prostatic cancer occurring with increasing frequency as men age, especially in the ages beyond 60 at a time when death from other factors often intervenes. Also, the spectrum of biologic aggressiveness of prostatic cancer is great, so that in some men following detection the tumor remains a latent histologic tumor and does not become clinically significant, whereas in other it progresses rapidly, metastasizes and kills the man in a relatively short 2-5 year period (1, 3).

In prostate cancer cells, two specific proteins that are made in very high concentrations are prostatic acid phosphatase (PAP) and prostate specific antigen (PSA) (4, 5, 6). These proteins have been characterized and have been used to follow response to therapy. With the development of cancer, the normal architecture of the gland becomes altered, including loss of the normal duct structure for the removal of secretions and thus the secretions reach the serum. Indeed measurement of serum PSA is suggested as a potential screening method for prostatic cancer. Indeed, the relative amount of PSA and/or PAP in the cancer reduces as compared to normal or benign tissue.

PAP was one of the earliest serum markers for detecting metastatic spread (4). PAP hydrolyses tyrosine phosphate and has a broad substrate specificity. Tyrosine phosphorylation is often increased with oncogenic transformation. It has been hypothesized that during neoplastic transformation there is less phosphatase activity available to inactivate proteins that are activated by phosphorylation on tyrosine residues. In some instances, insertion of phosphatases that have tyrosine phosphatase activity has reversed the malignant phenotype.

PSA is a protease and it is not readily appreciated how loss of its activity correlates with cancer development (5, 6). The proteolytic activity of PSA is inhibited by zinc. Zinc concentrations are high in the normal prostate and reduced in prostatic cancer. Possibly the loss of zinc allows for increased proteolytic activity by PSA. As proteases are involved in metastasis and some proteases stimulate mitotic activity, the potentially increased activity of PSA could be hypothesized to play a role in the tumors metastases and spread (7).

Both PSA and PAP are found in prostatic secretions. Both appear to be dependent on the presence of androgens for their production and are substantially reduced following androgen deprivation.

Prostate-specific membrane antigen (PSM) which appears to be localized to the prostatic membrane has been identified. This antigen was identified as the result of generating monoclonal antibodies to a prostatic cancer cell, LNCaP (8).

Dr. Horoszewicz established a cell line designated LNCaP from the lymph node of a hormone refractory,

heavily pretreated patient (9). This line was found to have an aneuploid human male karyotype. It maintained prostatic differentiation functionality in that it produced both PSA and PAP. It possessed an androgen receptor of high affinity and specificity. Mice were immunized with LNCaP cells and hybridomas were derived from sensitized animals. A monoclonal antibody was derived and was designated 7E11-C5 (8). The antibody staining was consistent with a membrane location and isolated fractions of LNCaP cell membranes exhibited a strongly positive reaction with immunoblotting and ELISA techniques. This antibody did not inhibit or enhance the growth of LNCaP cells in vitro or in vivo. The antibody to this antigen was remarkably specific to prostatic epithelial cells, as no reactivity was observed in any other component. Immunohistochemical staining of cancerous epithelial cells was more intense than that of normal or benign epithelial cells.

Dr. Horoszewicz also reported detection of immunoreactive material using 7E11-C5 in serum of prostatic cancer patients (8). The immunoreactivity was detectable in nearly 60% of patients with stage D-2 disease and in a slightly lower percentage of patients with earlier stage disease, but the numbers of patients in the latter group are small. Patients with benign prostatic hyperplasia (BPH) were negative. Patients with no apparent disease were negative, but 50-60% of patients in remission yet with active stable disease or with progression demonstrated positive serum reactivity. Patients with non prostatic tumors did not show immunoreactivity with 7E11-C5.

The 7E11-C5 monoclonal antibody is currently in clinical trials. The aldehyde groups of the antibody were oxidized and the linker-chelator glycol-tyrosyl-(n, ϵ-diethylenetriamine-pentacetic acid)-lysine (GYK-

DTPA) was coupled to the reactive aldehydes of the heavy chain (10). The resulting antibody was designated CYT-356. Immunohistochemical staining patterns were similar except that the CYT-356 modified antibody stained skeletal muscle. The comparison of CYT-356 with 7E11-C5 monoclonal antibody suggested both had binding to type 2 muscle fibers. The reason for the discrepancy with the earlier study, which reported skeletal muscle to be negative, was suggested to be due to differences in tissue fixation techniques. Still, the most intense and definite reaction was observed with prostatic epithelial cells, especially cancerous cells. Reactivity with mouse skeletal muscle was detected with immunohistochemistry but not in imaging studies. The $Indium^{111}$-labeled antibody localized to LNCaP tumors grown in nude mice with an uptake of nearly 30% of the injected dose per gram tumor at four days. In-vivo, no selective retention of the antibody was observed in antigen negative tumors such as PC-3 and DU-145, or by skeletal muscle. Very little was known about the PSM antigen. An effort at purification and characterization has been described at meetings by Dr. George Wright and colleagues (11, 12).

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Signal in lane 2 represent the 100kD PSM antigen. The EGFr was used as the positive control and is shown in lane 1. Incubation with rabbit antimouse (RAM) antibody alone served as negative control and is shown in lane 3.

Figures 2A-2D: Upper two photos show LNCaP cytospins staining positively for PSM antigen. Lower left in DU-145 and lower right is PC-3 cytospin, both negative for PSM antigen expression.

Figures 3A-3D: Upper two panels are human prostate sections (BPH) staining positively for PSM antigen. The lower two panels show invasive prostate carcinoma human sections staining positively for expression of the PSM antigen.

Figure 4: 100kD PSM antigen following immunoprecipitation of ^{35}S-Methionine labelled LNCaP cells with Cyt-356 antibody.

Figure 5: 3% agarose gels stained with Ethidium bromide revealing PCR products obtained using the degenerate PSM antigen primers. The arrow points to sample IN-20, which is a 1.1 kb PCR product which was later confirmed to be a partial cDNA coding for the PSM gene.

Figures 6A-6B: 2% agarose gels of plasmid DNA

resulting from TA cloning of PCR products. Inserts are excised from the PCR II vector (Invitrogen Corp.) by digestion with EcoRI. 1.1 kb PSM gene partial cDNA product is shown in lane 3 of gel 1.

Figure 7: Autoradiogram showing size of cDNA represented in applicants' LNCaP library using M-MLV reverse transcriptase.

Figure 8: Restriction analysis of full-length clones of PSM gene obtained after screening cDNA library. Samples have been cut with Not I and Sal I restriction enzymes to liberate the insert.

Figure 9: Plasmid Southern autoradiogram of full length PSM gene clones. Size is approximately 2.7 kb.

Figure 10: Northern blot revealing PSM expression limited to LNCaP prostate cancer line and H26 Ras-transfected LNCaP cell line. PC-3, DU-145, T-24, SKRC-27, HELA, MCF-7, HL-60, and others were are all negative.

Figure 11: Autoradiogram of Northern analysis revealing expression of 2.8 kb PSM message unique to the LNCaP cell line (lane 1), and absent from the DU-145 (lane 2) and PC-3 cell lines (lane 3). RNA size ladder is shown on the left (kb), and 28S and 18S ribosomal RNA

bands are indicated on the right.

Figures 12A-12B:
Results of PCR of human prostate tissues using PSM gene primers. Lanes are numbered from left to right. Lane 1, LNCaP; Lane 2, H26; Lane 3, DU-145; Lane 4, Normal Prostate; Lane 5, BPH; Lane 6, Prostate Cancer; Lane 7, BPH; Lane 8, Normal; Lane 9, BPH; Lane 10, BPH; Lane 11, BPH; Lane 12, Normal; Lane 13, Normal; Lane 14, Cancer; Lane 15, Cancer; Lane 16, Cancer; Lane 17, Normal; Lane 18, Cancer; Lane 19, IN-20 Control; Lane 20, PSM cDNA

Figure 13: Isoelectric point of PSM antigen (non-glycosylated)

Figures 14:1-8 Secondary structure of PSM antigen

Figures 15A-15B:
A. Hydrophilicity plot of PSM antigen
B. Prediction of membrane spanning segments

Figures 16:1-11
Homology with chicken, rat and human transferrin receptor sequence.

Figures 17A-17C:
Immunohistochemical detection of PSM antigen expression in prostate cell lines. Top panel reveals uniformly high level of expression in LNCaP cells; middle panel and lower panel are DU-145 and PC-3 cells respectively,

both negative.

Figure 18: Autoradiogram of protein gel revealing products of PSM coupled *in-vitro* transcription/translation. Non-glycosylated PSM polypeptide is seen at 84 kDa (lane 1) and PSM glycoprotein synthesized following the addition of microsomes is seen at 100 kDa (lane 2).

Figure 19: Western Blot analysis detecting PSM expression in transfected non-PSM expressing PC-3 cells. 100 kDa PSM glycoprotein species is clearly seen in LNCaP membranes (lane 1), LNCaP crude lysate (lane 2), and PSM-transfected PC-3 cells (lane 4), but is undetectable in native PC-3 cells (lane 3).

Figure 20: Autoradiogram of ribonuclease protection gel assaying for PSM mRNA expression in normal human tissues. Radiolabeled 1 kb DNA ladder (Gibco-BRL) is shown in lane 1. Undigested probe is 400 nucleotides (lane 2), expected protected PSM band is 350 nucleotides, and tRNA control is shown (lane 3). A strong signal is seen in human prostate (lane 11), with very faint, but detectable signals seen in human brain (lane 4) and human salivary gland (lane 12).

Figure 21: Autoradiogram of ribonuclease protection gel assaying for PSM mRNA expression in LNCaP tumors grown in

nude mice, and in human prostatic tissues. ^{32}P-labeled 1 kb DNA ladder is shown in lane 1. 298 nucleotide undigested probe is shown (lane 2), and tRNA control is shown (lane 3). PSM mRNA expression is clearly detectable in LNCaP cells (lane 4), orthotopically grown LNCaP tumors in nude mice with and without matrigel (lanes 5 and 6), and subcutaneously implanted and grown LNCaP tumors in nude mice (lane 7). PSM mRNA expression is also seen in normal human prostate (lane 8), and in a moderately differentiated human prostatic adenocarcinoma (lane 10). Very faint expression is seen in a sample of human prostate tissue with benign hyperplasia (lane 9).

Figure 22: Ribonuclease protection assay for PSM expression in LNCaP cells treated with physiologic doses of various steroids for 24 hours. ^{32}P-labeled DNA ladder is shown in lane 1. 298 nucleotide undigested probe is shown (lane 2), and tRNA control is shown (lane 3). PSM mRNA expression is highest in untreated LNCaP cells in charcoal-stripped media (lane 4). Applicant see significantly diminished PSM expression in LNCaP cells treated with DHT (lane 5), Testosterone (lane 6), Estradiol (lane 7), and Progesterone (lane 8), with little response to Dexamethasone (lane 9).

Figure 23: Data illustrating results of PSM DNA

and RNA presence in transfect Dunning cell lines employing Southern and Northern blotting techniques

Figures 24A-24B:

Figure A indicates the power of cytokine transfected cells to teach unmodified cells. Administration was directed to the parental flank or prostate cells. The results indicate the microenvironment considerations.

Figure B indicates actual potency at a particular site. The tumor was implanted in prostate cells and treated with immune cells at two different sites.

Figures 25A-25B:

Relates potency of cytokines in inhibiting growth of primary tumors. Animals administered un-modified parental tumor cells and administered as a vaccine transfected cells. Following prostatectomy of rodent tumor results in survival increase.

Figure 26: PCR amplification with nested primers improved the level of detection of prostatic cells from approximately one prostatic cell per 10,000 MCF-7 cells to better than one cell per million MCF-7 cells, using either PSA.

Figure 27: PCR amplification with nested primers improved the level of detection of prostatic cells from approximately one

prostatic cell per 10,000 MCF-7 cells to better than one cell per million MCF-7 cells, using PSM-derived primers.

Figure 28: A representative ethidium stained gel photograph for PSM-PCR. Samples run in lane A represent PCR products generated from the outer primers and samples in lanes labeled B are products of inner primer pairs.

Figure 29: PSM Southern blot autoradiograph. The sensitivity of the Southern blot analysis exceeded that of ethidium staining, as can be seen in several samples where the outer product is not visible on figure 3, but is detectable by Southern blotting as shown in figure 4.

Figure 30: Characteristics of the 16 patients analyzed with respect to their clinical stage, treatment, serum PSA and PAP values, and results of assay.

Figures 31A-31D:

The DNA sequence of the 3 kb XhoI fragment of p683 which includes 500 bp of DNA from the RNA start site was determined Sequence 683XFRVS starts from the 5' distal end of PSM promoter.

Figure 32: Potential binding sites on the PSM promoter.

Figure 33: Promoter activity of PSM up-stream fragment/CAT gene chimera.

Figure 34: Comparison between PSM and PSM′ cDNA. Sequence of the 5′ end of PSM cDNA (5) is shown. Underlined region denotes nucleotides which are present in PSM cDNA sequence but absent in PSM′ cDNA. Boxed region represents the putative transmembrane domain of PSM antigen. * Asterisk denotes the putative translation initiation site for PSM′.

Figure 35: Graphical representation of PSM and PSM′ cDNA sequences and antisense PSM RNA probe (b). PSM cDNA sequence with complete coding region (5). (a) PSM′ cDNA sequence from this study. (c) Cross hatched and open boxes denote sequences identity in PSM and PSM′. Hatched box indicates sequence absent from PSM′. Regions of cDNA sequence complementary to the antisense probe are indicated by dashed lines between the sequences.

Figure 36: RNase protection assay with PSM specific probe in primary prostatic tissues. Total cellular RNA was isolated from human prostatic samples: normal prostate, BPH, and CaP. PSM and PSM′ spliced variants are indicated with arrows at right. The left lane is a DNA ladder. Samples from different patients are classified as: lanes 3-6, *CaP*, carcinoma of prostate; *BPH*, benign prostatic hypertrophy, lanes 7-9; *normal*, normal prostatic tissue, lanes 10-12. Autoradiograph was exposed for longer period to read lanes 5 and 9.

Figure 37: Tumor Index, a quantification of the expression of PSM and PSM'. Expression of PSM and PSM' (Fig.3) was quantified by densitometry and expressed as a ratio of PSM/PSM' on the Y-axis. Three samples each were quantitated for primary CaP, BPH and normal prostate tissues. Two samples were quantitated for LNCaP. *Normal*, normal prostate tissue.

Figure 38: Characterization of PSM membrane bound and PSM' in the cytosol.

Figure 39: Intron 1F: Forward Sequence. Intron 1 contains a number of trinucleotide repeats which can be area associated with chromosomal instability in tumor cells. LNCaP cells and primary prostate tissue are identical, however in the PC-3 and Du-145 tumors they have substantially altered levels of these trinucleotide repeats which may relate to their lack of expression of PSM.

Figures 40A-40B:
Intron 1R: Reverse Sequence

Figure 41: Intron 2F: Forward Sequence

Figure 42: Intron 2R: Reverse Sequence

Figures 43A-43B:
Intron 3F: Forward Sequence

Figures 44A-44B:
Intron 3R: Reverse Sequence

Figures 45A-45B:

Intron 4F: Forward Sequence

Figures 46A-46B: Intron 4R: Reverse Sequence

Figures 47A-47D: Sequence of the genomic region upstream of the 5' transcription start site of PSM.

Figure 48: Photograph of ethidium bromide stained gel depicting representative negative and positive controls used in the study. Samples 1-5 were from, respectively: male with prostatis, a healthy female volunteer, a male with BPH, a control 1:1,000,000 dilution of LNCaP cells, and a patient with renal cell carcinoma. Below each reaction is the corresponding control reaction performed with beta-2-microglobulin primers to assure RNA integrity. No PCR products were detected for any of these negative controls.

Figure 49: Photograph of gel displaying representative positive PCR results using PSM primers in selected patients with either localized or disseminated prostate cancer. Sample 1-5 were from. respectively: a patient with clinically localized stage $T1_c$ disease, a radical prostatectomy patient with organ confined disease and a negative serum PSA, a radical prostatectomy patient with locally advanced disease and a negative serum PSA, a patient with

treated stage D2 disease, and a patient with treated hormone refractory disease.

Figure 50: Chromosomal location of PSM based on cosmid construction.

Figure 51: Human monochromosomal somatic cell hybrid blot showing that chromosome 11 contained the PSM genetic sequence by Southern analysis. DNA panel digested with PstI restriction enzyme and probed with PSM cDNA. Lanes M and H refer to mouse and hamster DNAs. The numbers correspond to the human chromosomal DNA in that hybrid.

Figure 52: Ribonuclease protection assay using PSM radiolabeled RNA probe revels an abundant PSM mRNA expression in AT6.1-11 clone 1, but not in AT6.1-11 clone 2, thereby mapping PSM to 11p11.2-13 region.

Figure 53: Tissue specific expression of PSM RNA by Northern blotting and RNAse protection assay.

Figure 54: Mapping of the PSM gene to the 11p11.2-p13 region of human chromosome 11 by southern blotting and in-situ hybridization.

Figure 55: Schematic of potential response elements.

Figure 56: Genomic organization of PSM gene.

Figure 57: Schematic of metastatic prostate cell

Figure 58A-58C:

Nucleic acid of PSM genomic DNA is read 5 prime away from the transcription start site: number on the sequences indicates nucleotide upstream from the start site. Therefore, nucleotide #121 is actually -121 using conventional numbering system.

Figure 59:

Representation of NAAG 1, acividin, azotomycin, and 6-diazo-5-oxo-norleucine, DON.

Figure 60:

Preparation of N-acetylaspartylglutamate, NAAG 1.

Figure 61:

Synthesis of N-acetylaspartylglutamate, NAAG 1.

Figure 62:

Synthesis of N-phosphonoacetylaspartyl-L-glutamate.

Figure 63:

Synthesis of 5-diethylphosphonon-2 amino benzylvalerate intermediate.

Figure 64:

Synthesis of analog 4 and 5.

Figure 65:

Representation of DON, analogs 17-20.

Figure 66:
Substrates for targeted drug delivery, analog 21 and 22.

Figure 67:
Dynemycin A and its mode of action.

Figure 68:
Synthesis of analog 28.

Figure 69:
Synthesis for intermediate analog 28.

Figure 70:
Attachment points for PALA.

Figure 71:
Mode of action for substrate 21.

Figures 72A-72D:
Intron 1F: Forward Sequence.

Figures 73A-73E:
Intron 1R: Reverse Sequence

Figures 74A-74C:
Intron 2F: Forward Sequence

Figures 75A-75C:
Intron 2R: Reverse Sequence

Figures 76A-76B:
Intron 3F: Forward Sequence

Figures 77A-77B:

Intron 3R: Reverse Sequence

Figures 78A-78C:

Intron 4F: Forward Sequence

Figures 79A-79E:

Intron 4RF: Reverse Sequence

Figure 80:

PSM genomic organization of the exons and 19 intron junction sequences. The exon/intron junctions (See Example 15) are as follows:

1. Exon /intron 1 at bp 389-390;
2. Exon /intron 2 at bp 490-491;
3. Exon /intron 3 at bp 681-682;
4. Exon /intron 4 at bp 784-785;
5. Exon /intron 5 at bp 911-912;
6. Exon /intron 6 at bp 1096-1097;
7. Exon /intron 7 at bp 1190-1191;
8. Exon /intron 8 at bp 1289- 1290;
9. Exon /intron 9 at bp 1375-1376;
10. Exon /intron 10 at bp 1496-1497;
11. Exon /intron 11 at bp 1579-1580;
12. Exon /intron 12 at bp 1640-1641;
13. Exon /intron 13 at bp 1708-1709;
14. Exon /intron 14 at bp 1803-1804;
15. Exon /intron 15 at bp 1892-1893;
16. Exon /intron 16 at bp 2158-2159;
17. Exon /intron 17 at bp 2240-2241;
18. Exon /intron 18 at bp 2334-2335;
19. Exon /intron 19 at bp 2644-2645.

SUMMARY OF THE INVENTION

This invention provides an isolated mammalian nucleic acid molecule encoding an alternatively spliced prostate-specific membrane (PSM') antigen.

This invention provides an isolated nucleic acid molecule encoding a prostate-specific membrane antigen promoter. This invention provides a method of detecting hematogenous micrometastic tumor cells of a subject, and determining prostate cancer progression in a subject.

Detailed Description of the Invention

Throughout this application, references to specific nucleotides are to nucleotides present on the coding strand of the nucleic acid. The following standard abbreviations are used throughout the specification to indicate specific nucleotides:

C=cytosine	A=adenosine
T=thymidine	G=guanosine

A "gene" means a nucleic acid molecule, the sequence of which includes all the information required for the normal regulated production of a particular protein, including the structural coding sequence, promoters and enhancers.

This invention provides an isolated mammalian nucleic acid encoding an alternatively spliced prostate-specific membrane (PSM') antigen.

This invention provides an isolated mammalian nucleic acid encoding a mammalian prostate-specific membrane (PSM) antigen.

This invention further provides an isolated mammalian DNA molecule of an isolated mammalian nucleic acid molecule encoding an alternatively spliced prostate-specific membrane antigen. This invention also provides an isolated mammalian cDNA molecule encoding a mammalian alternatively spliced prostate-specific membrane antigen. This invention provides an isolated mammalian RNA molecule encoding a mammalian alternatively spliced prostate-specific cytosolic antigen.

This invention further provides an isolated mammalian

DNA molecule of an isolated mammalian nucleic acid molecule encoding a mammalian prostate-specific membrane antigen. This invention also provides an isolated mammalian cDNA molecule encoding a mammalian prostate-specific membrane antigen. This invention provides an isolated mammalian RNA molecule encoding a mammalian prostate-specific membrane antigen.

In the preferred embodiment of this invention, the isolated nucleic sequence is cDNA from human as shown in Figures 47A-47D. This human sequence was submitted to GenBank (Los Alamos National Laboratory, Los Alamos, New Mexico) with Accession Number, M99487 and the description as PSM, Homo sapiens, 2653 base-pairs.

This invention also encompasses DNAs and cDNAs which encode amino acid sequences which differ from those of PSM or PSM' antigen, but which should not produce phenotypic changes. Alternatively, this invention also encompasses DNAs and cDNAs which hybridize to the DNA and cDNA of the subject invention. Hybridization methods are well known to those of skill in the art.

For example, high stringent hybridization conditions are selected at about 5° C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions will be those in which the salt concentration is at least about 0.02 molar at pH 7 and the temperature is at least about 60°C. As other factors may significantly affect the stringency of hybridization, including, among others, base composition and size of the complementary strands, the presence of organic solvents, ie. salt or formamide

concentration, and the extent of base mismatching, the combination of parameters is more important than the absolute measure of any one. For Example high stringency may be attained for example by overnight hybridization at about 68°C in a 6x SSC solution, washing at room temperature with 6x SSC solution, followed by washing at about 68°C in a 6x SSC in a 0.6x SSX solution.

Hybridization with moderate stringency may be attained for example by: 1) filter pre-hybridizing and hybridizing with a solution of 3x sodium chloride, sodium citrate (SSC), 50% formamide, 0.1M Tris buffer at Ph 7.5, 5x Denhardt's solution; 2.) pre-hybridization at 37°C for 4 hours; 3) hybridization at 37°C with amount of labelled probe equal to 3,000,000 cpm total for 16 hours; 4) wash in 2x SSC and 0.1% SDS solution; 5) wash 4x for 1 minute each at room temperature at 4x at 60°C for 30 minutes each; and 6) dry and expose to film.

The DNA molecules described and claimed herein are useful for the information which they provide concerning the amino acid sequence of the polypeptide and as products for the large scale synthesis of the polypeptide by a variety of recombinant techniques. The molecule is useful for generating new cloning and expression vectors, transformed and transfected prokaryotic and eukaryotic host cells, and new and useful methods for cultured growth of such host cells capable of expression of the polypeptide and related products.

Moreover, the isolated mammalian nucleic acid molecules encoding a mammalian prostate-specific membrane antigen and the alternatively spliced PSM' are useful for the development of probes to study the tumorigenesis of

prostate cancer.

This invention also provides an isolated nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of a nucleic acid molecule encoding the prostate-specific membrane antigen or the alternatively spliced prostate specific membrane antigen.

This nucleic acid molecule produced can either be DNA or RNA. As used herein, the phrase "specifically hybridizing" means the ability of a nucleic acid molecule to recognize a nucleic acid sequence complementary to its own and to form double-helical segments through hydrogen bonding between complementary base pairs.

This nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of a nucleic acid molecule encoding the prostate-specific membrane antigen can be used as a probe. Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary greatly in length and may be labeled with a detectable label, such as a radioisotope or fluorescent dye, to facilitate detection of the probe. DNA probe molecules may be produced by insertion of a DNA molecule which encodes PSM antigen into suitable vectors, such as plasmids or bacteriophages, followed by transforming into suitable bacterial host cells, replication in the transformed bacterial host cells and harvesting of the DNA probes, using methods well known in the art. Alternatively, probes may be generated chemically from DNA synthesizers.

RNA probes may be generated by inserting the PSM antigen molecule downstream of a bacteriophage promoter

such as T3, T7 or SP6. Large amounts of RNA probe may be produced by incubating the labeled nucleotides with the linearized PSM antigen fragment where it contains an upstream promoter in the presence of the appropriate RNA polymerase.

This invention also provides a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of a nucleic acid molecule which is complementary to the mammalian nucleic acid molecule encoding a mammalian prostate-specific membrane antigen. This molecule may either be a DNA or RNA molecule.

The current invention further provides a method of detecting the expression of a mammalian PSM or PSM' antigen expression in a cell which comprises obtaining total mRNA from the cell, contacting the mRNA so obtained with a labelled nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule encoding a mammalian PSM or PSM' antigen under hybridizing conditions, determining the presence of mRNA hybridized to the molecule and thereby detecting the expression of the mammalian prostate-specific membrane antigen in the cell. The nucleic acid molecules synthesized above may be used to detect expression of a PSM or PSM' antigen by detecting the presence of mRNA coding for the PSM antigen. Total mRNA from the cell may be isolated by many procedures well known to a person of ordinary skill in the art. The hybridizing conditions of the labelled nucleic acid molecules may be determined by routine experimentation well known in the art. The presence of mRNA hybridized to the probe may be determined by gel electrophoresis or other methods known in the art. By measuring the amount of the hybrid made, the expression of the PSM

antigen by the cell can be determined. The labeling may be radioactive. For an example, one or more radioactive nucleotides can be incorporated in the nucleic acid when it is made.

In one embodiment of this invention, nucleic acids are extracted by precipitation from lysed cells and the mRNA is isolated from the extract using an oligo-dT column which binds the poly-A tails of the mRNA molecules (13). The mRNA is then exposed to radioactively labelled probe on a nitrocellulose membrane, and the probe hybridizes to and thereby labels complementary mRNA sequences. Binding may be detected by luminescence autoradiography or scintillation counting. However, other methods for performing these steps are well known to those skilled in the art, and the discussion above is merely an example.

This invention further provides another method to detect expression of a PSM or PSM' antigen in tissue sections which comprises contacting the tissue sections with a labelled nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of nucleic acid molecules encoding a mammalian PSM antigen under hybridizing conditions, determining the presence of mRNA hybridized to the molecule and thereby detecting the expression of the mammalian PSM or PSM' antigen in tissue sections. The probes are also useful for in-situ hybridization or in order to locate tissues which express this gene, or for other hybridization assays for the presence of this gene or its mRNA in various biological tissues. The in-situ hybridization using a labelled nucleic acid molecule is well known in the art. Essentially, tissue sections are incubated with the labelled nucleic acid molecule to allow the hybridization to occur. The molecule will

carry a marker for the detection because it is "labelled", the amount of the hybrid will be determined based on the detection of the amount of the marker and so will the expression of PSM antigen.

This invention further provides isolated PSM or PSM' antigen nucleic acid molecule operatively linked to a promoter of RNA transcription. The isolated PSM or PSM' antigen sequence can be linked to vector systems. Various vectors including plasmid vectors, cosmid vectors, bacteriophage vectors and other viruses are well known to ordinary skilled practitioners. This invention further provides a vector which comprises the isolated nucleic acid molecule encoding for the PSM or PSM' antigen.

As an example to obtain these vectors, insert and vector DNA can both be exposed to a restriction enzyme to create complementary ends on both molecules which base pair with each other and are then ligated together with DNA ligase. Alternatively, linkers can be ligated to the insert DNA which correspond to a restriction site in the vector DNA, which is then digested with the restriction enzyme which cuts at that site. Other means are also available and known to an ordinary skilled practitioner.

In an embodiment, the PSM sequence is cloned in the Not I/Sal I site of pSPORT/vector (Gibco® - BRL). This plasmid, p55A-PSM, was deposited on August 14, 1992 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmid, p55A-PSM, was accorded ATCC Accession Number 75294.

This invention further provides a host vector system for the production of a polypeptide having the biological activity of the prostate-specific membrane antigen. These vectors may be transformed into a suitable host cell to form a host cell vector system for the production of a polypeptide having the biological activity of PSM antigen.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG (14). Similarly, a eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by methods well known in the art, for example the methods described above for constructing vectors in general. Expression vectors are useful to produce cells that express the PSM antigen.

This invention further provides an isolated DNA or cDNA molecule described hereinabove wherein the host cell is selected from the group consisting of bacterial cells (such as <u>E.coli</u>), yeast cells, fungal cells, insect cells and animal cells. Suitable animal cells include, but are not limited to Vero cells, HeLa cells, Cos cells, CV1 cells and various primary mammalian cells.

This invention further provides a method of producing a polypeptide having the biological activity of the prostate-specific membrane antigen which comprising

growing host cells of a vector system containing the PSM antigen sequence under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced.

This invention provides a mammalian cell comprising a DNA molecule encoding a mammalian PSM or PSM' antigen, such as a mammalian cell comprising a plasmid adapted for expression in a mammalian cell, which comprises a DNA molecule encoding a mammalian PSM antigen and the regulatory elements necessary for expression of the DNA in the mammalian cell so located relative to the DNA encoding the mammalian PSM or PSM' antigen as to permit expression thereof.

Numerous mammalian cells may be used as hosts, including, but not limited to, the mouse fibroblast cell NIH3T3, CHO cells, HeLa cells, Ltk$^-$ cells, Cos cells, etc. Expression plasmids such as that described supra may be used to transfect mammalian cells by methods well known in the art such as calcium phosphate precipitation, electroporation or DNA encoding the mammalian PSM antigen may be otherwise introduced into mammalian cells, e.g., by microinjection, to obtain mammalian cells which comprise DNA, e.g., cDNA or a plasmid, encoding a mammalian PSM antigen.

This invention provides a method for determining whether a ligand can bind to a mammalian prostate-specific membrane antigen which comprises contacting a mammalian cell comprising an isolated DNA molecule encoding a mammalian prostate-specific membrane antigen with the ligand under conditions permitting binding of ligands to the mammalian prostate-specific membrane antigen, and thereby determining whether the ligand binds to a mammalian prostate-specific membrane antigen.

This invention further provides ligands bound to the mammalian PSM or PSM' antigen.

This invention also provides a therapeutic agent comprising a ligand identified by the above-described method and a cytotoxic agent conjugated thereto. The cytotoxic agent may either be a radioisotope or a toxin. Examples of radioisotopes or toxins are well known to one of ordinary skill in the art.

This invention also provides a method of imaging prostate cancer in human patients which comprises administering to the patients at least one ligand identified by the above-described method, capable of binding to the cell surface of the prostate cancer cell and labelled with an imaging agent under conditions permitting formation of a complex between the ligand and the cell surface PSM or PSM' antigen. This invention further provides a composition comprising an effective imaging agent of the PSM OR PSM' antigen ligand and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known to one of ordinary skill in the art. For an example, such a pharmaceutically acceptable carrier can be physiological saline.

Also provided by this invention is a purified mammalian PSM and PSM' antigen. As used herein, the term "purified prostate-specific membrane antigen" shall mean isolated naturally-occurring prostate-specific membrane antigen or protein (purified from nature or manufactured such that the primary, secondary and tertiary conformation, and posttranslational modifications are identical to naturally-occurring material) as well as non-naturally occurring polypeptides having a primary structural conformation (i.e. continuous sequence of amino acid residues).

Such polypeptides include derivatives and analogs.

This invention provides an isolated nucleic acid molecule encoding a prostate-specific membrane antigen promoter. In one embodiment the PSM promoter has at least the sequence as in Figures 58A-58C.

This invention provides an isolated nucleic acid molecule encoding an alternatively spliced prostate-specific membrane antigen promoter.

This invention further provides a polypeptide encoded by the isolated mammalian nucleic acid sequence of PSM and PSM' antigen.

It is believed that there may be natural ligand interacting with the PSM or PSM' antigen. This invention provides a method to identify such natural ligand or other ligand which can bind to the PSM or PSM' antigen. A method to identify the ligand comprises a) coupling the purified mammalian PSM or PSM' antigen to a solid matrix, b) incubating the coupled purified mammalian PSM or PSM' protein with the potential ligands under the conditions permitting binding of ligands and the purified PSM or PSM' antigen; c) washing the ligand and coupled purified mammalian PSM or PSM' antigen complex formed in b) to eliminate the nonspecific binding and impurities and finally d) eluting the ligand from the bound purified mammalian PSM or PSM' antigen. The techniques of coupling proteins to a solid matrix are well known in the art. Potential ligands may either be deduced from the structure of mammalian PSM or PSM' by other empirical experiments known by ordinary skilled practitioners. The conditions for binding may also easily be determined and protocols for carrying such experimentation have long been well documented (15).

The ligand-PSM antigen complex will be washed. Finally, the bound ligand will be eluted and characterized. Standard ligands characterization techniques are well known in the art.

The above method may also be used to purify ligands from any biological source. For purification of natural ligands in the cell, cell lysates, serum or other biological samples will be used to incubate with the mammalian PSM or PSM' antigen bound on a matrix. Specific natural ligand will then be identified and purified as above described.

With the protein sequence information, antigenic areas may be identified and antibodies directed against these areas may be generated and targeted to the prostate cancer for imaging the cancer or therapies.

This invention provides an antibody directed against the amino acid sequence of a mammalian PSM or PSM' antigen.

This invention provides a method to select specific regions on the PSM or PSM' antigen to generate antibodies. The protein sequence may be determined from the PSM or PSM' DNA sequence. Amino acid sequences may be analyzed by methods well known to those skilled in the art to determine whether they produce hydrophobic or hydrophilic regions in the proteins which they build. In the case of cell membrane proteins, hydrophobic regions are well known to form the part of the protein that is inserted into the lipid bilayer of the cell membrane, while hydrophilic regions are located on the cell surface, in an aqueous environment. Usually, the hydrophilic regions will be more immunogenic than the hydrophobic regions. Therefore the hydrophilic amino acid

sequences may be selected and used to generate antibodies specific to mammalian PSM antigen. For an example, hydrophilic sequences of the human PSM antigen shown in hydrophilicity plot of Figures 16:1-11 may be easily selected. The selected peptides may be prepared using commercially available machines. As an alternative, DNA, such as a cDNA or a fragment thereof, may be cloned and expressed and the resulting polypeptide recovered and used as an immunogen.

Polyclonal antibodies against these peptides may be produced by immunizing animals using the selected peptides. Monoclonal antibodies are prepared using hybridoma technology by fusing antibody producing B cells from immunized animals with myeloma cells and selecting the resulting hybridoma cell line producing the desired antibody. Alternatively, monoclonal antibodies may be produced by <u>in vitro</u> techniques known to a person of ordinary skill in the art. These antibodies are useful to detect the expression of mammalian PSM antigen in living animals, in humans, or in biological tissues or fluids isolated from animals or humans.

In one embodiment, peptides Asp-Glu-Leu-Lys-Ala-Glu (SEQ ID No.), Asn-Glu-Asp-Gly-Asn-Glu (SEQ ID No.) and Lys-Ser-Pro-Asp-Glu-Gly (SEQ ID No.) of human PSM antigen are selected.

This invention further provides polyclonal and monoclonal antibody(ies) against peptides Asp-Glu-Leu-Lys-Ala-Glu (SEQ ID No.), Asn-Glu-Asp-Gly-Asn-Glu (SEQ ID No.) and Lys-Ser-Pro-Asp-Glu-Gly (SEQ ID No.).

This invention provides a therapeutic agent comprising antibodies or ligand(s) directed against PSM antigen

and a cytotoxic agent conjugated thereto or antibodies linked enzymes which activate prodrug to kill the tumor. The cytotoxic agent may either be a radioisotope or toxin.

This invention provides a method of imaging prostate cancer in human patients which comprises administering to the patient the monoclonal antibody directed against the peptide of the mammalian PSM or PSM' antigen capable of binding to the cell surface of the prostate cancer cell and labeled with an imaging agent under conditions permitting formation of a complex between the monoclonal antibody and the cell surface prostate-specific membrane antigen. The imaging agent is a radioisotope such as Indium^{111}.

This invention further provides a prostate cancer specific imaging agent comprising the antibody directed against PSM or PSM' antigen and a radioisotope conjugated thereto.

This invention also provides a composition comprising an effective imaging amount of the antibody directed against the PSM or PSM' antigen and a pharmaceutically acceptable carrier. The methods to determine effective imaging amounts are well known to a skilled practitioner. One method is by titration using different amounts of the antibody.

This invention further provides an immunoassay for measuring the amount of the prostate-specific membrane antigen in a biological sample comprising steps of a) contacting the biological sample with at least one antibody directed against the PSM or PSM' antigen to form a complex with said antibody and the prostate-specific membrane antigen, and b) measuring the amount of the prostate-specific membrane antigen in said

biological sample by measuring the amount of said complex. One example of the biological sample is a serum sample.

This invention provides a method to purify mammalian prostate-specific membrane antigen comprising steps of a) coupling the antibody directed against the PSM or PSM' antigen to a solid matrix; b) incubating the coupled antibody of a) with lysate containing prostate-specific membrane antigen under the condition which the antibody and prostate membrane specific can bind; c) washing the solid matrix to eliminate impurities and d) eluting the prostate-specific membrane antigen from the coupled antibody.

This invention also provides a transgenic nonhuman mammal which comprises the isolated nucleic acid molecule encoding a mammalian PSM or PSM' antigen. This invention further provides a transgenic nonhuman mammal whose genome comprises antisense DNA complementary to DNA encoding a mammalian prostate-specific membrane antigen so placed as to be transcribed into antisense mRNA complementary to mRNA encoding the prostate-specific membrane antigen and which hybridizes to mRNA encoding the prostate specific antigen thereby reducing its translation.

Animal model systems which elucidate the physiological and behavioral roles of mammalian PSM or PSM' antigen are produced by creating transgenic animals in which the expression of the PSM or PSM' antigen is either increased or decreased, or the amino acid sequence of the expressed PSM antigen is altered, by a variety of techniques. Examples of these techniques include, but are not limited to: 1) Insertion of normal or mutant versions of DNA encoding a mammalian PSM or PSM' antigen, by microinjection, electroporation, retroviral

transfection or other means well known to those skilled in the art, into appropriate fertilized embryos in order to produce a transgenic animal (16) or 2) Homologous recombination (17) of mutant or normal, human or animal versions of these genes with the native gene locus in transgenic animals to alter the regulation of expression or the structure of these PSM or PSM' antigen sequences. The technique of homologous recombination is well known in the art. It replaces the native gene with the inserted gene and so is useful for producing an animal that cannot express native PSM antigen but does express, for example, an inserted mutant PSM antigen, which has replaced the native PSM antigen in the animal's genome by recombination, resulting in undere xpression of the transporter. Microinjection adds genes to the genome, but does not remove them, and so is useful for producing an animal which expresses its own and added PSM antigens, resulting in over expression of the PSM antigens.

One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice are mated, and the resulting fertilized eggs are dissected out of their oviducts. The eggs are stored in an appropriate medium such as Me medium (16). DNA or cDNA encoding a mammalian PSM antigen is purified from a vector by methods well known in the art. Inducible promoters may be fused with the coding region of the DNA to provide an experimental means to regulate expression of the trans-gene. Alternatively or in addition, tissue specific regulatory elements may be fused with the coding region to permit tissue-specific expression of the trans-gene. The DNA, in an appropriately buffered solution, is put into a microinjection needle (which may be made from capillary tubing using a pipet puller) and the egg to be injected is put in a depression slide. The needle is inserted

into the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse (a mouse stimulated by the appropriate hormones to maintain pregnancy but which is not actually pregnant), where it proceeds to the uterus, implants, and develops to term. As noted above, microinjection is not the only method for inserting DNA into the egg cell, and is used here only for exemplary purposes.

Another use of the PSM antigen sequence is to isolate homologous gene or genes in different mammals. The gene or genes can be isolated by low stringency screening of either cDNA or genomic libraries of different mammals using probes from PSM sequence. The positive clones identified will be further analyzed by DNA sequencing techniques which are well known to an ordinary person skilled in the art. For example, the detection of members of the protein serine kinase family by homology probing.

This invention provides a method of suppressing or modulating metastatic ability of prostate tumor cells, prostate tumor growth or elimination of prostate tumor cells comprising introducing a DNA molecule encoding a prostate specific membrane antigen operatively linked to a 5′ regulatory element into a tumor cell of a subject, in a way that expression of the prostate specific membrane antigen is under the control of the regulatory element, thereby suppressing or modulating metastatic ability of prostate tumor cells, prostate tumor growth or elimination of prostate tumor cells. The subject may be a mammal or more specifically a human.

In one embodiment, the DNA molecule encoding prostate specific membrane antigen operatively linked to a 5′

regulatory element forms part of a transfer vector which is inserted into a cell or organism. In addition the vector is capable or replication and expression of prostate specific membrane antigen. The DNA molecule encoding prostate specific membrane antigen can be integrated into a genome of a eukaryotic or prokaryotic cell or in a host cell containing and/or expressing a prostate specific membrane antigen.

Further, the DNA molecule encoding prostate specific membrane antigen may be introduced by a bacterial, viral, fungal, animal, or liposomal delivery vehicle. Other means are also available and known to an ordinary skilled practitioner.

Further, the DNA molecule encoding a prostate specific membrane antigen operatively linked to a promoter or enhancer. A number of viral vectors have been described including those made from various promoters and other regulatory elements derived from virus sources. Promoters consist of short arrays of nucleic acid sequences that interact specifically with cellular proteins involved in transcription. The combination of different recognition sequences and the cellular concentration of the cognate transcription factors determines the efficiency with which a gene is transcribed in a particular cell type.

Examples of suitable promoters include a viral promoter. Viral promoters include: adenovirus promoter, an simian virus 40 (SV40) promoter, a cytomegalovirus (CMV) promoter, a mouse mammary tumor virus (MMTV) promoter, a Malony murine leukemia virus promoter, a murine sarcoma virus promoter, and a Rous sarcoma virus promoter.

Further, another suitable promoter is a heat shock

promoter. Additionally, a suitable promoter is a bacteriophage promoter. Examples of suitable bacteriophage promoters include but not limited to, a T7 promoter, a T3 promoter, an SP6 promoter, a lambda promoter, a baculovirus promoter.

Also suitable as a promoter is an animal cell promoter such as an interferon promoter, a metallothionein promoter, an immunoglobulin promoter. A fungal promoter is also a suitable promoter. Examples of fungal promoters include but are not limited to, an ADC1 promoter, an ARG promoter, an ADH promoter, a CYC1 promoter, a CUP promoter, an ENO1 promoter, a GAL promoter, a PHO promoter, a PGK promoter, a GAPDH promoter, a mating type factor promoter. Further, plant cell promoters and insect cell promoters are also suitable for the methods described herein.

This invention provides a method of suppressing or modulating metastatic ability of prostate tumor cells, prostate tumor growth or elimination of prostate tumor cells, comprising introducing a DNA molecule encoding a prostate specific membrane antigen operatively linked to a 5' regulatory element coupled with a therapeutic DNA into a tumor cell of a subject, thereby suppressing or modulating metastatic ability of prostate tumor cells, prostate tumor growth or elimination of prostate tumor cells. The subject may be a mammal or more specifically a human.

Further, the therapeutic DNA which is coupled to the DNA molecule encoding a prostate specific membrane antigen operatively linked to a 5' regulatory element into a tumor cell may code for a cytokine, viral antigen, or a pro-drug activating enzyme. Other means are also available and known to an ordinary skilled

practitioner.

In addition, this invention provides a prostate tumor cell, comprising a DNA molecule isolated from mammalian nucleic acid encoding a mammalian prostate-specific membrane antigen under the control of a prostate specific membrane antigen operatively linked to a 5' regulatory element.

As used herein, DNA molecules include complementary DNA (cDNA), synthetic DNA, and genomic DNA.

This invention provides a therapeutic vaccine for preventing human prostate tumor growth or stimulation of prostate tumor cells in a subject, comprising administering an effective amount to the prostate cell, and a pharmaceutical acceptable carrier, thereby preventing the tumor growth or stimulation of tumor cells in the subject. Other means are also available and known to an ordinary skilled practitioner.

This invention provides a method of detecting hematogenous micrometastic tumor cells of a subject, comprising (A) performing nested polymerase chain reaction (PCR) on blood, bone marrow or lymph node samples of the subject using the prostate specific membrane antigen primers or alternatively spliced prostate specific antigen primers, and (B) verifying micrometastases by DNA sequencing and Southern analysis, thereby detecting hematogenous micrometastic tumor cells of the subject. The subject may be a mammal or more specifically a human.

The micrometastatic tumor cell may be a prostatic cancer and the DNA primers may be derived from prostate specific antigen. Further, the subject may be administered with simultaneously an effective amount of

hormones, so as to increase expression of prostate specific membrane antigen. Further, growth factors or cytokine may be administered in separately or in conjunction with hormones. Cytokines include, but are not limited to: transforming growth factor beta, epidermal growth factor (EGF) family, fibroblast growth factors, hepatocyte growth factor, insulin-like growth factors, B-nerve growth factor, platelet-derived growth factor, vascular endothelial growth factor, interleukin 1, IL-1 receptor antagonist, interleukin 2, interleukin 3, interleukin 4, interleukin 5, interleukin 6, IL-6 soluble receptor, interleukin 7, interleukin 8, interleukin 9, interleukin 10, interleukin 11, interleukin 12, interleukin 13, angiogenin, chemokines, colony stimulating factors, granulocyte-macrophage colony stimulating factors, erythropoietin, interferon, interferon gamma, leukemia inhibitory factor, oncostatin M, pleiotrophin, secretory leukocyte protease inhibitor, stem cell factor, tumor necrosis factors, adhesion molecule, and soluble tumor necrosis factor (TNF) receptors.

This invention provides a method of abrogating the mitogenic response due to transferrin, comprising introducing a DNA molecule encoding prostate specific membrane antigen operatively linked to a 5' regulatory element into a tumor cell, the expression of which gene is directly associated with a defined pathological effect within a multicellular organism, thereby abrogating mitogen response due to transferrin. The tumor cell may be a prostate cell.

This invention provides a method of determining prostate cancer progression in a subject which comprises: a) obtaining a suitable prostate tissue sample; b) extracting RNA from the prostate tissue sample; c) performing a RNAse protection assay on the

RNA thereby forming a duplex RNA-RNA hybrid; d) detecting PSM and PSM' amounts in the tissue sample; e) calculating a PSM/PSM' tumor index, thereby determining prostate cancer progression in the subject. In-situ hyribridization may be performed in conjunction with the above detection method.

This invention provides a method of detecting prostate cancer in a subject which comprises: (a) obtaining from a subject a prostate tissue sample; (b) treating the tissue sample so as to separately recover nucleic acid molecules present in the prostate tissue sample; (c) contacting the resulting nucleic acid molecules with multiple pairs of single-stranded labeled oligonucleotide primers, each such pair being capable of specifically hybridizing to the tissue sample, under hybridizing conditions; (d) amplifying any nucleic acid molecules to which a pair of primers hybridizes so as to obtain a double-stranded amplification product; (e) treating any such double-stranded amplification product so as to obtain single-stranded nucleic acid molecules therefrom; (f) contacting any resulting single-stranded nucleic acid molecules with multiple single-stranded labeled oligonucleotide probes, each such probe containing the same label and being capable of specifically hybridizing with such tissue sample, under hybridizing conditions; (g) contacting any resulting hybrids with an antibody to which a marker is attached and which is capable of specifically forming a complex with the labeled-probe, when the probe is present in such a complex, under complexing conditions; and (h) detecting the presence of any resulting complexes, the presence thereof being indicative of prostate cancer in a subject.

This invention provides a method of enhancing antibody based targeting of PSM or PSM' in prostate tissue for

diagnosis or therapy of prostate cancer comprising administering to a patient b-FGF in sufficient amount to cause upregulation of PSM or PSM' expression.

This invention provides a method of enhancing antibody based targeting of PSM or PSM' in prostate tissue for diagnosis or therapy of prostate cancer comprising administering to a patient TGF in sufficient amount to cause upregulation of PSM expression or PSM'.

This invention provides a method of enhancing antibody based targeting of PSM or PSM' in prostate tissue for diagnosis or therapy of prostate cancer comprising administering to a patient EGF in sufficient amount to cause upregulation of PSM or PSM' expression.

This invention provides a pharmaceutical composition comprising an effective amount of PSM or the alternatively spliced PSM and a carrier or diluent. Further, this invention provides a method for administering to a subject, preferably a human, the pharmaceutical composition. Further, this invention provides a composition comprising an amount of PSM or the alternatively spliced PSM and a carrier or diluent. Specifically, this invention may be used as a food additive.

The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each subject.

Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by repeated doses at one or

more hour intervals by a subsequent injection or other administration.

As used herein administration means a method of administering to a subject. Such methods are well known to those skilled in the art and include, but are not limited to, administration topically, parenterally, orally, intravenously, intramuscularly, subcutaneously or by aerosol. Administration of PSM may be effected continuously or intermittently.

The pharmaceutical formulations or compositions of this invention may be in the dosage form of solid, semi-solid, or liquid such as, *e.g.*, suspensions, aerosols or the like. Preferably the compositions are administered in unit dosage forms suitable for single administration of precise dosage amounts. The compositions may also include, depending on the formulation desired, pharmaceutically-acceptable, non-toxic carriers or diluents, which are defined as vehicles commonly used to formulate pharmaceutical compositions for animal or human administration. The diluent is selected so as not to affect the biological activity of the combination. Examples of such diluents are distilled water, physiological saline, Ringer's solution, dextrose solution, and Hank's solution. In addition, the pharmaceutical composition or formulation may also include other carriers, adjuvants; or nontoxic, nontherapeutic, nonimmunogenic stabilizers and the like. Effective amounts of such diluent or carrier are those amounts which are effective to obtain a pharmaceutically acceptable formulation in terms of solubility of components, or biological activity, etc

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the

specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

EXPERIMENTAL DETAILS

EXAMPLE 1:

Materials and Methods: The approach for cloning the gene involved purification of the antigen by immunoprecipitation, and microsequencing of several internal peptides for use in synthesizing degenerate oligonucleotide primers for subsequent use in the polymerase chain reaction (19, 20). A partial cDNA was amplified as a PCR product and this was used as a homologous probe to clone the full-length cDNA molecule from a LNCaP (Lymph Node Carcinoma of Prostate) cell line cDNA plasmid library (8).

Western Analysis of the PSM Antigen: Membrane proteins were isolated from cells by hypotonic lysis followed by centrifugation over a sucrose density gradient (21). 10-20μg of LNCaP, DU-145, and PC-3 membrane proteins were electrophoresed through a 10% SDS-PAGE resolving gel with a 4% stacking gel at 9-10 milliamps for 16-18 hours. Proteins were electroblotted onto PVDF membranes (Millipore® Corp.) in transfer buffer (48mM Tris base, 39mM Glycine, 20% Methanol) at 25 volts overnight at 4°C. Membranes were blocked in TSB (0.15M NaCl, 0.01M Tris base, 5% BSA) for 30 minutes at room temperature followed by incubation with 10-15μg/ml of CYT-356 monoclonal antibody (Cytogen Corp.) for 2 hours. Membranes were then incubated with 10-15μg/ml of rabbit anti-mouse immunoglobulin (Accurate Scientific) for 1 hour at room temperature followed by incubation with ^{125}I-Protein A (Amersham®) at 1×10^6 cpm/ml at room temperature. Membranes were then washed and autoradiographed for 12-24 hours at -70°C (Figure 1).

Immunohistochemical Analysis of PSM Antigen Expression: The avidin-biotin method of immunohistochemical detection was employed to analyze both human tissue sections and cell lines for PSM Antigen expression (22). Cryostat-cut prostate tissue sections (4-6μm thick) were fixed in methanol/acetone for 10 minutes. Cell cytospins were made on glass slides using 50,000 cells/100μl/slide. Samples were treated with 1% hydrogen peroxide in PBS for 10-15 minutes in order to remove any endogenous peroxidase activity. Tissue sections were washed several times in PBS, and then incubated with the appropriate suppressor serum for 20 minutes. The suppressor serum was drained off and the sections or cells were then incubated with the diluted CYT-356 monoclonal antibody for 1 hour. Samples were then washed with PBS and sequentially incubated with secondary antibodies (horse or goat immunoglobulins, 1:200 dilution for 30 minutes), and with avidin-biotin complexes (1:25 dilution for 30 minutes). DAB was used as a chromogen, followed by hematoxylin counterstaining and mounting. Frozen sections of prostate samples and duplicate cell cytospins were used as controls for each experiment. As a positive control, the anti-cytokeratin monoclonal antibody CAM 5.2 was used following the same procedure described above. Tissue sections are considered by us to express the PSM antigen if at least 5% of the cells demonstrate immunoreactivity. The scoring system is as follows: 1 = <5%; 2 = 5-19%; 3 = 20-75%; and 4 = >75% positive cells. Homogeneity versus heterogeneity was accounted for by evaluating positive and negative cells in 3-5 high power light microscopic fields (400x), recording the percentage of positive cells among 100-500 cells. The intensity of immunostaining is graded on a 1+ to 4+ scale, where 1+ represents mild, 2-3+ represents moderate, and 4+ represents intense immunostaining as compared to positive controls.

Immunoprecipitation of the PSM Antigen: 80%-confluent LNCaP cells in 100mm petri dishes were starved in RPMI media without methionine for 2 hours, after which ^{35}S-Methionine was added at 100μCi/ml and the cells were grown for another 16-18 hours. Cells were then washed and lysed by the addition of 1ml of lysis buffer (1% Triton X-100, 50mM Hepes pH 7.5, 10% glycerol, 150mM $MgCl_2$, 1mM PMSF, and 1mM EGTA) with incubation for 20 minutes at 4°C. Lysates were pre-cleared by mixing with Pansorbin® cells (Calbiochem®) for 90 minutes at 4°C. Cell lysates were then mixed with Protein A Sepharose® CL-4B beads (Pharmacia®) previously bound with CYT-356 antibody (Cytogen Corp.) and RAM antibody (Accurate Scientific) for 3-4 hours at 4°C. 12μg of antibody was used per 3mg of beads per petri dish. Beads were then washed with HNTG buffer (20mM Hepes pH 7.5, 150mM NaCl, 0.1% Triton X-100, 10% glycerol, and 2mM Sodium Orthovanadate), resuspended in sample loading buffer containing ß-mercaptoethanol, denatured at 95°C for 5-10 minutes and run on a 10% SDS-PAGE gel with a 4° stacking gel at 10 milliamps overnight. Gels were stained with Coomassie Blue, destained with acetic acid/methanol, and dried down in a vacuum dryer at 60°C. Gels were then autoradiographed for 16-24 hours at -70°C (Figures 2A-2D).

Immunoprecipitation and Peptide Sequencing: The procedure described above for immunoprecipitation was repeated with 8 confluent petri dishes containing approximately $6x10^7$ LNCaP cells. The immunoprecipitation product was pooled and loaded into two lanes of a 10% SDS-PAGE gel and electrophoresed at 9-10 milliamps for 16 hours. Proteins were electroblotted onto Nitrocellulose BA-85 membranes (Schleicher and Schuell®) for 2 hours at 75 volts at 4°C in transfer buffer. Membranes were stained with Ponceau Red to visualize the proteins and the 100kD

protein band was excised, solubilized, and digested proteolytically with trypsin. HPLC was then performed on the digested sample on an Applied Biosystems Model 171C and clear dominant peptide peaks were selected and sequenced by modified Edman degradation on a modified post liquid Applied Biosystems Model 477A Protein/Peptide Microsequencer (23). Sequencing data on all of the peptides is included within this document. The amino-terminus of the PSM antigen was sequenced by a similar method which involved purifying the antigen by immunoprecipitation and transfer via electro-blotting to a PVDF membrane (Millipore®). Protein was analyzed on an Applied Biosystems Model 477A Protein/Peptide Sequencer and the amino terminus was found to be <u>blocked</u>, and therefore no sequence data could be obtained by this technique.

PSM Antigen Peptide Sequences:

2T17	#5	SLYES(W)TK (SEQ ID No.)
2T22	#9	(S)YPDGXNLPGG(g)VQR (SEQ ID No.)
2T26	#3	FYDPMFK (SEQ ID No.)
2T27	#4	IYNVIGTL(K) (SEQ ID No.)
2T34	#6	FLYXXTQIPHLAGTEQNFQLAK (SEQ ID No.)
2T35	#2	G/PVILYSDPADYFAPD/GVK (SEQ ID No.)
2T38	#1	AFIDPLGLPDRPFYR (SEQ ID No.)
2T46	#8	YAGESFPGIYDALFDIESK (SEQ ID No.)
2T47	#7	TILFAS(W)DAEEFGXX(q)STE(e)A(E)... (SEQ ID No.)

Notes: X means that no residue could be identified at this position. Capital denotes identification but with a lower degree of confidence. (lower case) means residue present but at very low levels. ... indicates sequence continues but has dropped below detection limit.

All of these peptide sequences were verified to be unique after a complete homology search of the translated Genbank computer database.

Degenerate PCR: Sense and anti-sense 5'-unphosphorylated degenerate oligonucleotide primers 17 to 20 nucleotides in length corresponding to portions of the above peptides were synthesized on an Applied Biosystems Model 394A DNA Synthesizer. These primers have degeneracies from 32 to 144. The primers used are shown below. The underlined amino acids in the peptides represent the residues used in primer design.

Peptide 3: **FYDPMFK** (SEQ ID No.)

PSM Primer "A" TT(C or T) - TA(C or T) - GA(C or T) - CCX - ATG - TT (SEQ ID No.)

PSM Primer "B" AAC - ATX - GG(A or G) - TC(A or G) - TA(A or G) - AA (SEQ ID No.)

Primer A is sense primer and B is anti-sense. Degeneracy is 32-fold.

Peptide 4: **IYNVIGTL(K)** (SEQ ID No. 6)

PSM Primer "C" AT(T or C or A) - TA(T or C) - AA(T or C) - GTX - AT(T or C or A) - GG (SEQ ID No.)

PSM Primer "D" CC(A or T or G) - ATX - AC(G or A) - TT(A or G) - TA(A or G or T) - AT (SEQ ID No.)

Primer C is sense primer and D is anti-sense. Degeneracy is 144-fold.

Peptide 2: **G/PVILYSDPADYFAPD/GVK** (SEQ ID No.)

PSM Primer "E" CCX - GCX - GA(T or C) - TA(T or C) - TT(T or C) - GC (SEQ ID No.)

PSM Primer "F" GC(G or A) - AA(A or G) - TA(A or G) - TXC - GCX - GG (SEQ ID No.)

Primer E is sense primer and F is antisense primer. Degeneracy is 128-fold.

Peptide 6: **FLYXXTQIPHLAGTEQNFQLAK** (SEQ ID No.)

PSM Primer "I" ACX - GA(A or G) - CA(A or G) - AA(T or C) - TT(T or C) - CA(A or G) - CT (SEQ ID No.)

PSM Primer "J" AG - (T or C)TG - (A or G)AA - (A or G)TT - (T or C)TG - (T or C)TC - XGT (SEQ ID No.)

PSM Primer "K" GA(A or G) - CA(A or G) - AA(T or C) - TT(T or C) CA(A or G) - CT (SEQ ID No.)

PSM Primer "L" AG - (T or C)TG - (A or G)AA - (A or G)TT - (T or C)TG - (T or C)TC (SEQ ID No. 22)

Primers I and K are sense primers and J and L are anti-sense. I and J have degeneracies of 128-fold and K and L have 32-fold degeneracy.

Peptide 7: **TILFAS(W)DAEEFGXX(q)STE(e)A(E)...** (SEQ ID No.)

PSM Primer "M" TGG - GA(T or C) - GCX - GA(A or G) - GA(A or G) - TT(C or T) - GG (SEQ ID No.)

PSM Primer "N" CC - (G or A)AA - (T or C)TC - (T or C)TC - XGC - (A or G)TC - CCA (SEQ ID No.)

PSM Primer "O" TGG - GA(T or C) - GCX - GA(A or G) -

GA(A or G) - TT (SEQ ID No.)

PSM Primer "P" AA - (T or C)TC - (T or C)TC - XGC - (A or G)TC - CCA (SEQ ID No.)

Primers M and O are sense primers and N and P are anti-sense. M and N have degeneracy of 64-fold and O and P are 32-fold degenerate.

Degenerate PCR was performed using a Perkin-Elmer Model 480 DNA thermal cycler. cDNA template for the PCR was prepared from LNCaP mRNA which had been isolated by standard methods of oligo dT chromatography (Collaborative Research). The cDNA synthesis was carried out as follows:

4.5μl	LNCaP poly A+ RNA (2μg)
1.0μl	Oligo dT primers (0.5μg)
<u>4.5μl</u>	<u>dH_2O</u>
10μl	

Incubate at 68°C x 10 minutes.
Quick chill on ice x 5 minutes.

<u>Add:</u>

4μl	5 x RT Buffer
2μl	0.1M DTT
1μl	10mM dNTPs
0.5μl	RNasin (Promega)
<u>1.5μl</u>	<u>dH_2O</u>
19μl	

Incubate for 2 minutes at 37°C.
Add 1μl Superscript® Reverse Transcriptase (Gibco®-BRL)
Incubate for 1 hour at 37°C.

Add 30μl dH_2O.
Use 2μl per PCR reaction.

Degenerate PCR reactions were optimized by varying the annealing temperatures, Mg++ concentrations, primer concentrations, buffer composition, extension times and number of cycles. The optimal thermal cycler profile was: Denaturation at 94°C x 30 seconds, Annealing at 45-55°C for 1 minute (depending on the mean T_m of the primers used), and Extension at 72°C for 2 minutes.

5μl	10 x PCR Buffer*
5μl	2.5mM dNTP Mix
5μl	Primer Mix (containing 0.5-1.0μg each of sense and anti-sense primers)
5μl	100mM ß-mercaptoethanol
2μl	LNCaP cDNA template
5μl	25mM $MgCl_2$ (2.5mM final)
21μl	dH_2O
2μl	diluted Taq Polymerase (0.5U/μl)
50μl total volume	

Tubes were overlaid with 60μl of light mineral oil and amplified for 30 cycles. PCR products were analyzed by electrophoresing 5μl of each sample on a 2-3% agarose gel followed by staining with Ethidium bromide and photography.

*10x PCR Buffer
166mM NH_4SO_4
670mM Tris, pH 8.8
2mg/ml BSA

Representative photographs displaying PCR products are shown in Figure 5.

Cloning of PCR Products: In order to further analyze

these PCR products, these products were cloned into a suitable plasmid vector using "TA Cloning" (Invitrogen® Corp.). The cloning strategy employed here is to directly ligate PCR products into a plasmid vector possessing overhanging T residues at the insertion site, exploiting the fact that Taq polymerase leaves overhanging A residues at the ends of the PCR products. The ligation mixes are transformed into competent E. coli cells and resulting colonies are grown up, plasmid DNA is isolated by the alkaline lysis method (24), and screened by restriction analysis (Figures 6A-6B).

DNA Sequencing of PCR Products: TA Clones of PCR products were then sequenced by the dideoxy method (25) using Sequenase (U.S. Biochemical). 3-4μg of each plasmid DNA was denatured with NaOH and ethanol precipitated. Labeling reactions were carried out as per the manufacturers recommendations using ^{35}S-ATP, and the reactions were terminated as per the same protocol. Sequencing products were then analyzed on 6% polyacrylamide/7M Urea gels using an IBI sequencing apparatus. Gels were run at 120 watts for 2 hours. Following electrophoresis, the gels were fixed for 15-20 minutes in 10% methanol/10% acetic acid, transferred onto Whatman 3MM paper and dried down in a Biorad® vacuum dryer at 80°C for 2 hours. Gels were then autoradiographed at room temperature for 16-24 hours. In order to determine whether the PCR products were the correct clones, the sequences obtained at the 5' and 3' ends of the molecules were analyzed for the correct primer sequences, as well as adjacent sequences which corresponded to portions of the peptides not used in the design of the primers.

IN-20 was confirmed to be correct and represent a partial cDNA for the PSM gene. In this PCR reaction, I and N primers were used. The DNA sequence reading

from the I primer was:

ACG GAG CAA AAC TTT CAG CTT GCA AAG (SEQ ID No.)
<u>T E Q N F Q</u> L A K (SEQ ID No.)

The underlined amino acids were the portion of peptide 6 that was used to design this sense primer and the remaining amino acids which agree with those present within the peptide confirm that this end of the molecule represents the correct protein (PSM antigen).

When analyzed the other end of the molecule by reading from the N primer the anti-sense sequence was:

CTC TTC GGC ATC CCA GCT TGC AAA CAA AAT TGT TCT (SEQ ID No.)

Sense (complementary) Sequence:

AGA ACA ATT TTG TTT GCA AGC TGG GAT GCC AAG GAG (SEQ ID No.)
R T I L F A S <u>W D A E E</u> (SEQ ID No.)

The underlined amino acids here represent the portion of peptide 7 used to create primer N. All of the amino acids upstream of this primer are correct in the IN-20 clone, agreeing with the amino acids found in peptide 7. Further DNA sequencing has enabled us to identify the presence of other PSM peptides within the DNA sequence of the positive clone.

The DNA sequence of this partial cDNA was found to be unique when screened on the Genbank computer database.

cDNA Library Construction and Cloning of Full - Length PSM cDNA: A cDNA library from LNCaP mRNA was

constructed using the Superscript® plasmid system (BRL®-Gibco). The library was transformed using competent DH5-α cells and plated onto 100mm plates containing LB plus 100μg/ml of Carbenicillin. Plates were grown overnight at 37°C and colonies were transferred to nitrocellulose filters. Filters were processed and screened as per Grunstein and Hogness (26), using the 1.1kb partial cDNA homologous probe which was radiolabelled with ^{32}P-dCTP by random priming (27). Eight positive colonies were obtained which upon DNA restriction and sequencing analysis proved to represent full-length cDNA molecules coding for the PSM antigen. Shown in Figure 7 is an autoradiogram showing the size of the cDNA molecules represented in the library and in Figure 8 restriction analysis of several full-length clones is shown. Figure 9 is a plasmid Southern analysis of the samples in Figure 8, showing that they all hybridize to the 1.1kb partial cDNA probe.

Both the cDNA as well as the antigen have been screened through the Genbank Computer database (Human Genome Project) and have been found to be unique.

Northern Analysis of PSM Gene Expression: Northern analysis (28) of the PSM gene has revealed that expression is limited to the prostate and to prostate carcinoma.

RNA samples (either 10μg of total RNA or 2μg of poly A+ RNA) were denatured and electrophoresed through 1.1% agarose/formaldehyde gels at 60 milliamps for 6-8 hours. RNA was then transferred to Nytran® nylon membranes (Schleicher and Schuell®) by pressure blotting in 10x SSC with a Posi-blotter (Stratagene®). RNA was cross-linked to the membranes using a Stratalinker (Stratagene®) and subsequently baked in a

vacuum oven at 80°C for 2 hours. Blots were prehybridized at 65°C for 2 hours in prehybridization solution (BRL®) and subsequently hybridized for 16 hours in hybridization buffer (BRL®) containing 1-2 x 10^6 cpm/ml of ^{32} P-labelled random-primed cDNA probe. Membranes were washed twice in 1x SSPE/1% SDS and twice in 0.1x SSPE/1% SDS at 42°C. Membranes were then air-dried and autoradiographed for 12-36 hours at -70°C.

PCR Analysis of PSM Gene Expression in Human Prostate Tissues: PCR was performed on 15 human prostate samples to determine PSM gene expression. Five samples each from normal prostate tissue, benign prostatic hyperplasia, and prostate cancer were used (histology confirmed by MSKCC Pathology Department).

10μg of total RNA from each sample was reverse transcribed to made cDNA template as previously described in section IV. The primers used corresponded to the 5' and 3' ends of the 1.1kb partial cDNA, IN-20, and therefore the expected size of the amplified band is 1.1kb. Since the T_m of the primers is 64°C. PCR primers were annealed at 60°C. PCR was carried out for 35 cycles using the same conditions previously described in section IV.

LNCaP and H26 - Ras transfected LNCaP (29) were included as a positive control and DU-145 as a negative control. 14/15 samples clearly amplified the 1.1kb band and therefore express the gene.

Experimental Results

The gene which encodes the 100kD PSM antigen has been identified. The complete cDNA sequence is shown in Sequence ID #1. Underneath that nucleic acid sequence is the predicted translated amino acid sequence. The total number of the amino acids is 750, ID #2. The

hydrophilicity of the predicted protein sequence is shown in Figures 16:1-11. Shown in Figures 17A-17C are three peptides with the highest point of hydrophilicity. They are: Asp-Glu-Leu-Lys-Ala-Glu (SEQ ID No.); Asn-Glu-Asp-Gly-Asn-Glu (SEQ ID No. ; and Lys-Ser-Pro-Asp-Glu-Gly (SEQ ID No.).

By the method of Klein, Kanehisa and DeLisi, a specific membrane-spanning domain is identified. The sequence is from the amino acid #19 to amino acid #44: Ala-Gly-Ala-Leu-Val-Leu-Aal-Gly-Gly-Phe-Phe-Leu-Leu-Gly-Phe-Leu-Phe (SEQ ID No.).

This predicted membrane-spanning domain was computed on PC Gene (computer software program). This data enables prediction of inner and outer membrane domains of the PSM antigen which aids in designing antibodies for uses in targeting and imaging prostate cancer.

When the PSM antigen sequence with other known sequences of the GeneBank were compared, homology between the PSM antigen sequence and the transferrin receptor sequence were found. The data are shown in Figure 18.

Experimental Discussions

Potential Uses for PSM Antigen:

1. Tumor detection:

Microscopic:

Unambiguous tumor designation can be accomplished by use of probes for different antigens. For prostatic cancer, the PSM antigen probe may prove beneficial. Thus PSM could be used for diagnostic purposes and this could be accomplished at the microscopic level using in-situ hybridization using sense (control) and

antisense probes derived from the coding region of the cDNA cloned by the applicants. This could be used in assessment of local extraprostatic extension, involvement of lymph node, bone or other metastatic sites. As bone metastasis presents a major problem in prostatic cancer, early detection of metastatic spread is required especially for staging. In some tumors detection of tumor cells in bone marrow portends a grim prognosis and suggests that interventions aimed at metastasis be tried. Detection of PSM antigen expression in bone marrow aspirates or sections may provide such early information. PCR amplification or in-situ hybridization may be used. Using RT-PCR cells in the circulating can be detected by hematogenous metastasis.

2. Antigenic site identification

The knowledge of the cDNA for the antigen also provides for the identification of areas that would serve as good antigens for the development of antibodies for use against specific amino acid sequences of the antigen. Such sequences may be at different regions such as outside, membrane or inside of the PSM antigen. The development of these specific antibodies would provide for immunohistochemical identification of the antigen. These derived antibodies could then be developed for use, especially ones that work in paraffin fixed sections as well as frozen section as they have the greatest utility for immunodiagnosis.

3. Restriction fragment length polymorphism and genomic DNA

Restriction fragment length polymorphisms (RFLPS) have proven to be useful in documenting the progression of genetic damage that occurs during tumor initiation and promotion. It may be that RFLP analysis will demonstrate that changes in PSM sequence restriction

mapping may provide evidence of predisposition to risk or malignant potential or progression of the prostatic tumor.

Depending on the chromosomal location of the PSM antigen, the PSM antigen gene may serve as a useful chromosome location marker for chromosome analysis.

4. Serum

With the development of antigen specific antibodies, if the antigen or selected antigen fragments appear in the serum they may provide for a serum marker for the presence of metastatic disease and be useful individually or in combination with other prostate specific markers.

5. Imaging

As the cDNA sequence implies that the antigen has the characteristics of a membrane spanning protein with the majority of the protein on the exofacial surface, antibodies, especially monoclonal antibodies to the peptide fragments exposed and specific to the tumor may provide for tumor imaging local extension of metastatic tumor or residual tumor following prostatectomy or irradiation. The knowledge of the coding region permits the generation of monoclonal antibodies and these can be used in combination to provide for maximal imaging purposes. Because the antigen shares a similarity with the transferrin receptor based on cDNA analysis (approximately 54%), it may be that there is a specific normal ligand for this antigen and that identification of the ligand(s) would provide another means of imaging.

6. Isolation of ligands

The PSM antigen can be used to isolate the normal ligand(s) that bind to it. These ligand(s) depending

on specificity may be used for targeting, or their serum levels may be predictive of disease status. If it is found that the normal ligand for PSM is a carrier molecule then it may be that PSM could be used to bind to that ligand for therapy purposes (like an iron chelating substance) to help remove the ligand from the circulation. If the ligand promotes tumor growth or metastasis then providing soluble PSM antigen would remove the ligand from binding the prostate. Knowledge of PSM antigen structure could lend to generation of small fragment that binds ligand which could serve the same purpose.

7. Therapeutic uses

a) Ligands. The knowledge that the cDNA structure of PSM antigen shares structural homology with the transferrin receptor (54% on the nucleic acid level) implies that there may be an endogenous ligand for the receptor that may or may not be transferrin-like. Transferrin is thought to be a ligand that transports iron into the cell after binding to the transferrin receptor. However, apotransferrin is being reported to be a growth factor for some cells which express the transferrin receptor (30). Whether transferrin is a ligand for this antigen or some other ligand binds to this ligand remains to be determined. If a ligand is identified it may carry a specific substance such as a metal ion (iron or zinc or other) into the tumor and thus serve as a means to deliver toxic substances (radioactive or cytotoxic chemical i.e. toxin like ricin or cytotoxic alkylating agent or cytotoxic prodrug) to the tumor.

The main metastatic site for prostatic tumor is the bone. The bone and bone stroma are rich in transferrin. Recent studies suggest that this microenvironment is what provides the right "soil" for

prostatic metastasis in the bone (31). It may be that this also promotes attachment as well, these factors which reduce this ability may diminish prostatic metastasis to the bone and prostatic metastatic growth in the bone.

It was found that the ligand for the new antigen (thought to be an oncogene and marker of malignant phenotype in breast carcinoma) served to induce differentiation of breast cancer cells and thus could serve as a treatment for rather than promotor of the disease. It may be that ligand binding to the right region of PSM whether with natural ligand or with an antibody may serve a similar function.

Antibodies against PSM antigen coupled with a cytotoxic agent will be useful to eliminate prostate cancer cells. Transferrin receptor antibodies with toxin conjugates are cytotoxic to a number of tumor cells as tumor cells tend to express increased levels of transferrin receptor (32). Transferrin receptors take up molecules into the cell by endocytosis. Antibody drug combinations can be toxic. Transferrin linked toxin can be toxic.

b) Antibodies against PSM antigen coupled with a cytotoxic agent will be useful to eliminate prostate cancer cells. The cytotoxic agent may be a radioisotope or toxin as known in ordinary skill of the art. The linkage of the antibody and the toxin or radioisotope can be chemical. Examples of direct linked toxins are doxorubicin, chlorambucil, ricin, pseudomonas exotoxin etc., or a hybrid toxin can be generated ½ with specificity for PSM and the other ½ with specificity for the toxin. Such a bivalent molecule can serve to bind to the tumor and the other ½ to deliver a cytotoxic to the tumor or to bind to and

activate a cytotoxic lymphocyte such as binding to the T_1 - ζ receptor complex. Antibodies of required specificity can also be cloned into T cells and by replacing the immunoglobulin domain of the T cell receptor (TcR); cloning in the desired MAb heavy and light chains; splicing the U_h and U_L gene segments with the constant regions of the α and ß TCR chains and transfecting these chimeric Ab/TcR genes in the patients' T cells, propagating these hybrid cells and infusing them into the patient (33). Specific knowledge of tissue specific antigens for targets and generation of MAb's specific for such targets will help make this a usable approach. Because the PSM antigen coding region provides knowledge of the entire coding region, it is possible to generate a number of antibodies which could then be used in combination to achieve an additive or synergistic anti-tumor action. The antibodies can be linked to enzymes which can activate non-toxic prodrugs at its site of the tumor such as Ab-carboxypeptidase and 4-(bis(2 chloroethyl)amino)benzoyl-α-glutamic acid and its active parent drug in mice (34).

It is possible to produce a toxic genetic chimera such as TP-40 a genetic recombinant that possesses the cDNA from TGF-alpha and the toxic portion of pseudomonas exotoxin so the TGF and portion of the hybrid binds the epidermal growth factor receptor (EGFR) and the pseudomonas portion gets taken up into the cell enzymatically and inactivates the ribosomes ability to perform protein synthesis resulting in cell death.

In addition, once the ligand for the PSM antigen is identified, toxin can be chemically conjugated to the ligands. Such conjugated ligands can be therapeutically useful. Examples of the toxins are daunomycin, chlorambucil, ricin, pseudomonas exotoxin,

etc. Alternatively, chimeric construct can be created linking the cDNA of the ligand with the cDNA of the toxin. An example of such toxin is TGFα and pseudomonas exotoxin (35).

8. Others

The PSM antigen may have other uses. It is well known that the prostate is rich in zinc, if the antigen provides function relative to this or other biologic function the PSM antigen may provide for utility in the treatment of other prostatic pathologies such as benign hyperplastic growth and/or prostatitis.

Because purified PSM antigen can be generated, the purified PSM antigen can be linked to beads and use it like a standard "affinity" purification. Serum, urine or other biological samples can be used to incubate with the PSM antigen bound onto beads. The beads may be washed thoroughly and then eluted with salt or pH gradient. The eluted material is SDS gel purified and used as a sample for microsequencing. The sequences will be compared with other known proteins and if unique, the technique of degenerated PCR can be employed for obtaining the ligand. Once known, the affinity of the ligand will be determined by standard protocols (15).

References of Example 1

1. Chiaroda, A. (1991) National roundtable of prostate cancer: research directions. Cancer Res. 51: 2498-2505.

2. Coffey, D.S. Prostate Cancer - An overview of an increasing dilemma. Cancer Supplement, *71,3:* 880-886, 1993.

3. Warner, J.A., et al., (1991) Future developments of non-hormonal systemic therapy for prostatic carcinoma. Urologic Clin. North Amer. 18:25-33.

4. Nguyen, L., et al., (1990) Prostatic acid phosphatase in the serum of cancer patients with prostatic cancer is a specific phosphotyrosine acid phosphatase. Clin. Chem. 35:1450-1455.

5. Henttu, P., et al., (1989) cDNA coding for the entire human prostate specific antigen show high homologies to the human tissue kallikrein genes. Bioch. Biophys. Res. Comm. 160:903-908.

6. Yong, CY-F., et al., (1991) Hormonal regulation of prostate-specific antigen messenger RNA in human prostatic adenocarcinoma cell line LNCaP. Cancer Res. 51:3748-3752.

7. Liotta, L.A. (1986) Tumor invasion and metastases: role of the extracellular matrix. Cancer Res. 46:1-7.

8. Horoszewicz, J.S., et al. (1987) Monoclonal antibodies to a new antigenic marker in epithelial prostatic cells and serum of prostatic cancer patients. Anticancer Res. 7:927-936.

9. Horoszewicz, J.S., et al. (1983) LNCaP model of human prostatic carcinoma. Cancer Res., 43:1809-1818.

10. Lopes, D., et al. (1990) Immunohistochemical and pharmacokinetic characterization of the site-specific immunoconjugate CYT-356, derived from anti-prostate monoclonal antibody 7E11-C5. Cancer Res., 50:6423-6429.

11. Wright, Jr., et al., (1990) Characterization of a new carcinoma associated marker:7E11-C5. Antibod. Immunoconj. Radiopharm.3:(abst#193).

12. Feng, Q., et al., (1991) Purification and biochemical characterization of the 7E11-C5 prostate carcinoma associated antigen. Proc. Amer. Assoc. Cancer Res. 32:239.

13. Axelrod, H.R., et al., Preclinical results and human immunohistochemical studies with ^{90}Y-CYT-356. A New prostate cancer agent. Abstract 596. AUA 87th Annual Meeting, May 10-14, 1992. Washington, D.C.

14. Maniatis, T., et al., (1982) Molecular Cloning; Cold Spring Harbor Laboratory, pp.197-98 (1982).

15. Maniatis, et al., (1982) Molecular Cloning, Cold Spring Harbor Laboratory.

16. Methods in Enzymology vol. 34: 1-810, 1974 (E) B. Jacoby and M. Wilchek Academic Press, New York 1974.

17. Hogan B. et al. (1986) Manipulating the Mouse Embryo, A Laboratory Manual, Cold Spring Harbor

Laboratory.

18. Capecchi M.R. Science (1989) 244:1288-1292; Zimmer, A. and Gruss, P. (1989) Nature 338:150-153.

19. Trowbridge, I.S., (1982) Prospects for the clinical use of cytotoxic monoclonal antibodies conjugates in the treatment of cancer. Cancer Surveys 1:543-556.

20. Hank, S.K. (1987) Homology probing: Identification of cDNA clones encoding members of the protein-serine kinase family. Proc. Natl. Acad. Sci. 84:388-392.

21. Lee, C.C., et al., (1988) Generation of cDNA probes directed by amino acid sequences: cloning of urate oxidase. Science, 239, 1288.

22. Girgis, S.I., et al. (1988) Generation of DNA probes for peptides with highly degenerate codons using mixed primer PCR. Nucleic Acids Res. 16:10932.

23. Kartner, N., et al. (1977) Isolation of plasma membranes from human skin fibroblasts. J. Membrane Biology, 36:191-211.

24. Hsu, S.M., et al. (1981) Comparative study of the immunoperoxidase, anti-peroxidase, and avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. Am. J. Pathology, 75:734.

25. Tempst, P., et al. (1989) Examination of automated polypeptide sequencing using standard

phenylisothiocyanate reagent and subpicomole high performance liquid chromatography analysis. Analytical Biochem. 183:290-300.

26. Birnboim, H.C. (1983) A rapid alkaline extraction method for the isolation of plasmid DNA. Meth. Enzymol, 100:243-255.

27. Sanger, F., et al. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA, 74:5463-5467.

28. Grunstein, M., et al. (1975) Colony hybridization as a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA, 72:3961.

29. Feinberg, A.P., et al. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem, 132, 6.

30. Rave, N., et al. (1979) Identification of procollagen mRNAs transferred to diazobenzylomethyl paper from formaldehyde gels. Nucleic Acids Research, 6:3559.

31. Voeller, H.J., et al. (1991) v-ras^{H} expression confers hormone-independent in-vitro growth to LNCaP prostate carcinoma cells. Molec. Endocrinology. Vol. 5. No. 2, 209-216.

32. Sirbasku, D.A. (1991) Purification of an equine apotransferrin variant (thyromedin) essential for thyroid hormone dependent growth of GH_1, rat pituitary tumor cells in chemically defined culture. Biochem., 30:295-301.

33. Rossi, M.C. (1992) Selective stimulation of prostatic carcinoma cell proliferation by transferrin. Proc. Natl. Acad. Sci. (USA) 89:6197-6201.

34. Eshhan, Z. (1990) Chimeric T cell receptor which incorporates the anti-tumor specificity of a monoclonal antibody with the cytolytic activity of T cells: a model system for immunotherapeutic approach. B. J. Cancer 62:27-29.

35. Antonie, P. (1990) Disposition of the prodrug 4-(bis(2 chloroethyl) amino)benzoyl-α-glutamic acid and its active parent in mice. B. J. Cancer 62:905-914.

36. Heimbrook, D.C., et al. (1990) Transforming growth factor alpha-pseudomonas exotoxin fusion protein prolongs survival of nude mice bearing tumor xenografts. Proc. Natl. Acad. Sci. (USA) 87:4697-4701.

37. Chiarodo, A. National Cancer Institute roundtable on prostate cancer; future research directions. Cancer Res., *51:* 2498-2505, 1991.

38. Abdel-Nabi, H., Wright, G.L., Gulfo, J.V., Petrylak, D.P., Neal, C.E., Texter, J.E., Begun, F.P., Tyson, I., Heal, A., Mitchell, E., Purnell, G., and Harwood, S.J. Monoclonal antibodies and radioimmunoconjugates in the diagnosis and treatment of prostate cancer. Semin. Urol., *10:* 45-54, 1992.

EXAMPLE 2:

EXPRESSION OF THE PROSTATE SPECIFIC MEMBRANE ANTIGEN

A 2.65 kb complementary DNA encoding PSM was cloned. Immunohistochemical analysis of the LNCaP, DU-145, and PC-3 prostate cancer cell lines for PSM expression using the 7E11-C5.3 antibody reveals intense staining in the LNCaP cells, with no detectable expression in both the DU-145 and PC-3 cells. Coupled *in-vitro* transcription/ translation of the 2.65 kb full-length PSM cDNA yields an 84 kDa protein corresponding to the predicted polypeptide molecular weight of PSM. Post-translational modification of this protein with pancreatic canine microsomes yields the expected 100 kDa PSM antigen. Following transfection of PC-3 cells with the full-length PSM cDNA in a eukaryotic expression vector applicant's detect expression of the PSM glycoprotein by Western analysis using the 7E11-C5.3 monoclonal antibody. Ribonuclease protection analysis demonstrates that the expression of PSM mRNA is almost entirely prostate-specific in human tissues. PSM expression appears to be highest in hormone-deprived states and is hormonally modulated by steroids, with DHT downregulating PSM expression in the human prostate cancer cell line LNCaP by 8-10 fold, testosterone downregulating PSM by 3-4 fold, and corticosteroids showing no significant effect. Normal and malignant prostatic tissues consistently show high PSM expression, whereas heterogeneous, and at times absent, from expression of PSM in benign prostatic hyperplasia. LNCaP tumors implanted and grown both orthotopically and subcutaneously in nude mice, abundantly express PSM providing an excellent *in-vivo* model system to study the regulation and modulation of PSM expression.

Materials and Methods:

Cells and Reagents: The LNCaP, DU-145, and PC-3 cell lines were obtained from the American Type Culture Collection. Details regarding the establishment and characteristics of these cell lines have been previously published (5A,7A,8A). Unless specified otherwise, LNCaP cells were grown in RPMI 1640 media supplemented with L-glutamine, nonessential amino acids, and 5% fetal calf serum (Gibco-BRL, Gaithersburg, MD.) in a CO_2 incubator at 37C. DU-145 and PC-3 cells were grown in minimal essential medium supplemented with 10% fetal calf serum. All cell media were obtained from the MSKCC Media Preparation Facility. Restriction and modifying enzymes were purchased from Gibco-BRL unless otherwise specified.

Immunohistochemical Detection of PSM: Avidin-biotin method of detection was employed to analyze prostate cancer cell lines for PSM antigen expression (9A). Cell cytospins were made on glass slides using $5x10^4$ cells/100ul per slide. Slides were washed twice with PBS and then incubated with the appropriate suppressor serum for 20 minutes. The suppressor serum was drained off and the cells were incubated with diluted 7E11-C5.3 (5g/ml) monoclonal antibody for 1 hour. Samples were then washed with PBS and sequentially incubated with secondary antibodies for 30 minutes and with avidin-biotin complexes for 30 minutes. Diaminobenzidine served as the chromogen and color development followed by hematoxylin counterstaining and mounting. Duplicate cell cytospins were used as controls for each experiment. As a positive control, the anti-cytokeratin monoclonal antibody CAM 5.2 was used following the same procedure described above. Human EJ bladder carcinoma cells served as a negative control.

In-Vitro Transcription/Translation of PSM Antigen: Plasmid 55A containing the full length 2.65 kb PSM cDNA in the plasmid pSPORT 1 (Gibco-BRL) was transcribed in-vitro using the Promega TNT system (Promega Corp. Madison, WI). T7 RNA polymerase was added to the cDNA in a reaction mixture containing rabbit reticulocyte lysate, an amino acid mixture lacking methionine, buffer, and ^{35}S-Methionine (Amersham) and incubated at 30C for 90 minutes. Post-translational modification of the resulting protein was accomplished by the addition of pancreatic canine microsomes into the reaction mixture (Promega Corp. Madison, WI.). Protein products were analyzed by electrophoresis on 10% SDS-PAGE gels which were subsequently treated with Amplify autoradiography enhancer (Amersham, Arlington Heights, IL.) according to the manufacturers instructions and dried at 80C in a vacuum dryer. Gels were autoradiographed overnight at -70C using Hyperfilm MP (Amersham).

Transfection of PSM into PC-3 Cells: The full length PSM cDNA was subcloned into the pREP7 eukaryotic expression vector (Invitrogen, San Diego, CA.). Plasmid DNA was purified from transformed DH5-alpha bacteria (Gibco-BRL) using Qiagen maxi-prep plasmid isolation columns (Qiagen Inc., Chatsworth, CA.). Purified plasmid DNA (6-10g) was diluted with 900ul of Optimem media (Gibco-BRL) and mixed with 30ul of Lipofectin reagent (Gibco-BRL) which had been previously diluted with 9001 of Optimem media. This mixture was added to T-75 flasks of 40-50% confluent PC-3 cells in Optimem media. After 24-36 hours, cells were trypsinized and split into 100mm dishes containing RPMI 1640 media supplemented with 10% fetal calf serum and 1 mg/ml of Hygromycin B (Calbiochem, La Jolla, CA.). The dose of Hygromycin B used was previously determined by a time course/dose response

cytotoxicity assay. Cells were maintained in this media for 2-3 weeks with changes of media and Hygromycin B every 4-5 days until discrete colonies appeared. Colonies were isolated using 6mm cloning cylinders and expanded in the same media. As a control, PC-3 cells were also transfected with the pREP7 plasmid alone. RNA was isolated from the transfected cells and PSM mRNA expression was detected by both RNase Protection analysis (described later) and by Northern analysis.

Western Blot Detection of PSM Expression: Crude protein lysates were isolated from LNCaP, PC-3, and PSM-transfected PC-3 cells as previously described (10A). LNCaP cell membranes were also isolated according to published methods (10A). Protein concentrations were quantitated by the Bradford method using the BioRad protein reagent kit (BioRad, Richmond, CA.). Following denaturation, 20μg of protein was electrophoresed on a 10% SDS-PAGE gel at 25 mA for 4 hours. Gels were electroblotted onto Immobilon P membranes (Millipore, Bedford, MA.) overnight at 4C. Membranes were blocked in 0.15M NaCl/0.01M Tris-HCl (TS) plus 5% BSA followed by a 1 hour incubation with 7E11-C5.3 monoclonal antibody (10μg/ml). Blots were washed 4 times with 0.15M NaCl/0.01M Tris-HCl/0.05% Triton-X 100 (TS-X) and incubated for 1 hour with rabbit anti-mouse IgG (Accurate Scientific, Westbury, N.Y.) at a concentration of 10μg/ml.

Blots were then washed 4 times with TS-X and labeled with ^{125}I-Protein A (Amersham, Arlington Heights, IL.) at a concentration of 1 million cpm/ml. Blots were then washed 4 times with TS-X and dried on Whatman 3MM paper, followed by overnight autoradiography at -70C using Hyperfilm MP (Amersham).

Orthotopic and Subcutaneous LNCaP Tumor Growth in Nude Mice: LNCaP cells were harvested from sub-confluent cultures by a one minute exposure to a solution of 0.25% trypsin and 0.02% EDTA. Cells were resuspended in RPMI 1640 media with 5% fetal bovine serum, washed and diluted in either Matrigel (Collaborative Biomedical Products, Bedford, MA.) or calcium and magnesium-free Hank's balanced salt solution (HBSS). Only single cell suspensions with greater than 90% viability by trypan blue exclusion were used for in vivo injection. Male athymic Swiss (nu/nu) nude mice 4-6 weeks of age were obtained from the Memorial Sloan-Kettering Cancer Center Animal Facility. For subcutaneous tumor cell injection one million LNCaP cells resuspended in 0.2 mls. of Matrigel were injected into the hindlimb of each mouse using a disposable syringe fitted with a 28 gauge needle. For orthotopic injection, mice were first anesthetized with an intraperitoneal injection of Pentobarbital and placed in the supine position. The abdomen was cleansed with Betadine and the prostate was exposed through a midline incision. 2.5 million LNCaP tumor cells in 0.1 ml. were injected directly into either posterior lobe using a 1 ml disposable syringe and a 28 gauge needle. LNCaP cells with and without Matrigel were injected. Abdominal closure was achieved in one layer using Autoclip wound clips (Clay Adams, Parsippany, N.J.). Tumors were harvested in 6-8 weeks, confirmed histologically by faculty of the Memorial Sloan-Kettering Cancer Center Pathology Department, and frozen in liquid nitrogen for subsequent RNA isolation.

RNA Isolation: Total cellular RNA was isolated from cells and tissues by standard techniques (11,12) as well as by using RNAzol B (Cinna/Biotecx, Houston, TX.). RNA concentrations and quality were assessed by UV spectroscopy on a Beckman DU 640 spectrophotometer

and by gel analysis. Human tissue total RNA samples were purchased from Clontech Laboratories, Inc., Palo Alto, CA.

Ribonuclease Protection Assays: A portion of the PSM cDNA was subcloned into the plasmid vector pSPORT 1 (Gibco-BRL) and the orientation of the cDNA insert relative to the flanking T7 and SP6 RNA polymerase promoters was verified by restriction analysis. Linearization of this plasmid upstream of the PSM insert followed by transcription with SP6 RNA polymerase yields a 400 nucleotide antisense RNA probe, of which 350 nucleotides should be protected from RNase digestion by PSM RNA. This probe was used in Figure 20. Plasmid IN-20, containing a 1 kb partial PSM cDNA in the plasmid pCR II (Invitrogen) was also used for riboprobe synthesis. IN-20 linearized with Xmn I (Gibco-BRL) yields a 298 nucleotide anti-sense RNA probe when transcribed using SP6 RNA polymerase, of which 260 nucleotides should be protected from RNase digestion by PSM mRNA. This probe was used in Figures 21 and 22. Probes were synthesized using SP6 RNA polymerase (Gibco-BRL), rNTPs (Gibco-BRL), RNAsin (Promega), and ^{32}P-rCTP (NEN, Wilmington, DE.) according to published protocols (13). Probes were purified over NENSORB 20 purification columns (NEN) and approximately 1 million cpm of purified, radiolabeled PSM probe was mixed with 10μ of each RNA and hybridized overnight at 45C using buffers and reagents from the RPA II kit (Ambion, Austin, TX). Samples were processed as per manufacturer's instructions and analyzed on 5% polyacrilamide/7M urea denaturing gels using Seq ACRYL reagents (ISS, Natick, MA.). Gels were pre-heated to 55C and run for approximately 1-2 hours at 25 watts. Gels were then fixed for 30 minutes in 10% methanol/10% acetic acid, dried onto Whatman 3MM paper at 80C in a BioRad vacuum dryer and autoradiographed overnight with

Hyperfilm MP (Amersham). Quantitation of PSM expression was determined by using a scanning laser densitometer (LKB, Piscataway, NJ.).

Steroid Modulation Experiment: LNCaP cells (2 million) were plated onto T-75 flasks in RPMI 1640 media supplemented with 5% fetal calf serum and grown 24 hours until approximately 30-40% confluent. Flasks were then washed several times with phophate-buffered saline and RPMI medium supplemented with 5% charcoal-extracted serum was added. Cells were then grown for another 24 hours, at which time dihydrotesterone, testosterone, estradiol, progesterone, and dexamethasone (Steraloids Inc., Wilton, NH.) were added at a final concentration of 2 nM. Cells were grown for another 24 hours and RNA was then harvested as previously described and PSM expression analyzed by ribonuclease protection analysis.

<u>Experimental Results</u>

Immunohistochemical Detection of PSM: Using the 7E11-C5.3 anti-PSM monoclonal antibody, PSM expression is clearly detectable in the LNCaP prostate cancer cell line, but not in the PC-3 and DU-145 cell lines (Figures 17A-17C). All normal and malignant prostatic tissues analyzed stained positively for PSM expression.

In-Vitro Transcription/Translation of PSM Antigen: As shown in Figure 18, coupled in-vitro transcription/translation of the 2.65 kb full-length PSM cDNA yields an 84 kDa protein species in agreement with the expected protein product from the 750 amino acid PSM open reading frame. Following post-translational modification using pancreatic canine microsomes were obtained a 100 kDa glycosylated protein species

consistent with the mature, native PSM antigen.

Detection of PSM Antigen in LNCaP Cell Membranes and Transfected PC-3 Cells: PC-3 cells transfected with the full length PSM cDNA in the pREP7 expression vector were assayed for expression of SM mRNA by Northern analysis. A clone with high PSM mRNA expression was selected for PSM antigen analysis by Western blotting using the 7E11-C5.3 antibody. In Figure 19, the 100 kDa PSM antigen is well expressed in LNCaP cell lysate and membrane fractions, as well as in PSM-transfected PC-3 cells but not in native PC-3 cells. This detectable expression in the transfected PC-3 cells proves that the previously cloned 2.65 kb PSM cDNA encodes the antigen recognized by the 7E11-C5.3 anti-prostate monoclonal antibody.

PSM mRNA Expression: Expression of PSM mRNA in normal human tissues was analyzed using ribonuclease protection assays. Tissue expression of PSM appears predominantly within the prostate, with very low levels of expression detectable in human brain and salivary gland (Figure 20). No detectable PSM mRNA expression was evident in non-prostatic human tissues when analyzed by Northern analysis. On occasion it is noted that detectable PSM expression in normal human small intestine tissue, however this mRNA expression is variable depending upon the specific riboprobe used. All samples of normal human prostate and human prostatic adenocarcinoma assayed have revealed clearly detectable PSM expression, whereas generally decreased or absent expression of PSM in tissues exhibiting benign hyperplasia (Figure 21). In human LNCaP tumors grown both orthotopically and subcutaneously in nude mice abundant PSM expression with or without the use of matrigel, which is required for the growth of subcutaneously implanted LNCaP cells was detected

(Figure 21). PSM mRNA expression is distinctly modulated by the presence of steroids in physiologic doses (Figure 22). DHT downregulated expression by 8-10 fold after 24 hours and testosterone diminished PSM expression by 3-4 fold. Estradiol and progesterone also downregulated PSM expression in LNCaP cells, perhaps as a result of binding to the mutated androgen receptor known to exist in the LNCaP cell. Overall, PSM expression is highest in the untreated LNCaP cells grown in steroid-depleted media, a situation that simulates the hormone-deprived (castrate) state *in-vivo*. This experiment was repeated at steroid dosages ranging from 2-200 nM and at time points from 6 hours to 7 days with similar results; maximal downregulation of PSM mRNA was seen with DHT at 24 hours at doses of 2-20 nM.

<u>Experimental Discussion</u>

Previous research has provided two valuable prostatic bio-markers, PAP and PSA, both of which have had a significant impact on the diagnosis, treatment, and management of prostate malignancies. The present work describing the preliminary characterization of the prostate-specific membrane antigen (PSM) reveals it to be a gene with many interesting features. PSM is almost entirely prostate-specific as are PAP and PSA, and as such may enable further delineation of the unique functions and behavior of the prostate. The predicted sequence of the PSM protein (3) and its presence in the LNCaP cell membrane as determined by Western blotting and immunohistochemistry, indicate that it is an integral membrane protein. Thus, PSM provides an attractive cell surface epitope for antibody-directed diagnostic imaging and cytotoxic targeting modalities (14). The ability to synthesize the PSM antigen *in-vitro* and to produce tumor

xenografts maintaining high levels of PSM expression provides us with a convenient and attractive model system to further study and characterize the regulation and modulation of PSM expression. Also, the high level of PSM expression in the LNCaP cells provides an excellent *in-vitro* model system. Since PSM expression is hormonally-responsive to steroids and may be highly expressed in hormone-refractory disease (15). The detection of PSM mRNA expression in minute quantities in brain, salivary gland, and small intestine warrants further investigation, although these tissues were negative for expression of PSM antigen by immunohistochemistry using the 7E11-C5.3 antibody (16). In all of these tissues, particularly small intestine, mRNA expression using a probe corresponding to a region of the PSM cDNA near the 3' end, whereas expression when using a 5' end PSM probe was not detected. These results may indicate that the PSM mRNA transcript undergoes alternative splicing in different tissues.

Applicants approach is based on prostate tissue specific promotor: enzyme or cytokine chimeras. Promotor specific activation of prodrugs such as non toxic gancyclovir which is converted to a toxic metabolite by herpes simplex thymidine kinase or the prodrug 4-(bis(2chloroethyl)amino)benzoyl-1-glutamic acid to the benzoic acid mustard alkylating agent by the pseudomonas carboxy peptidase G2 was examined. As these drugs are activated by the enzyme (chimera) specifically in the tumor the active drug is released only locally in the tumor environment, destroying the surrounding tumor cells. Promotor specific activation of cytokines such as IL-12, IL-2 or GM-CSF for activation and specific antitumor vaccination is examined. Lastly the tissue specific promotor activation of cellular death genes may also prove to be useful in this area.

Gene Therapy Chimeras: The establishment of "chimeric DNA" for gene therapy requires the joining of different segments of DNA together to make a new DNA that has characteristics of both precursor DNA species involved in the linkage. In this proposal the two pieces being linked involve different functional aspects of DNA, the promotor region which allows for the reading of the DNA for the formation of mRNA will provide specificity and the DNA sequence coding for the mRNA will provide for therapeutic functional DNA.

DNA-Specified Enzyme or Cytokine mRNA: When effective, antitumor drugs can cause the regression of very large amounts of tumor. The main requirements for antitumor drug activity is the requirement to achieve both a long enough time (t) and high enough concentration (c) (cxt) of exposure of the tumor to the toxic drug to assure sufficient cell damage for cell death to occur. The drug also must be "active" and the toxicity for the tumor greater than for the hosts normal cells (22). The availability of the drug to the tumor depends on tumor blood flow and the drugs diffusion ability. Blood flow to the tumor does not provide for selectivity as blood flow to many normal tissues is often as great or greater than that to the tumor. The majority of chemotherapeutic cytotoxic drugs are often as toxic to normal tissue as to tumor tissue. Dividing cells are often more sensitive than non-dividing normal cells, but in many slow growing solid tumors such as prostatic cancer this does not provide for antitumor specificity (22).

Previously a means to increase tumor specificity of antitumor drugs was to utilize tumor associated enzymes to activate nontoxic prodrugs to cytotoxic agents (19). A problem with this approach was that most of the enzymes found in tumors were not totally specific in

their activity and similar substrate active enzymes or the same enzyme at only slightly lower amounts was found in other tissue and thus normal tissues were still at risk for damage.

To provide absolute specificity and unique activity, viral, bacterial and fungal enzymes which have unique specificity for selected prodrugs were found which were not present in human or other animal cells. Attempts to utilize enzymes such as herpes simplex thymidine kinase, bacterial cytosine deaminase and carboxypeptidase G-2 were linked to antibody targeting systems with modest success (19). Unfortunately, antibody targeted enzymes limit the number of enzymes available per cell. Also, most antibodies do not have a high tumor target to normal tissue ratio thus normal tissues are still exposed reducing the specificity of these unique enzymes. Antibodies are large molecules that have poor diffusion properties and the addition of the enzymes molecular weight further reduces the antibodies diffusion.

Gene therapy could produce the best desired result if it could achieve the specific expression of a protein in the tumor and not normal tissue in order that a high local concentration of the enzyme be available for the production in the tumor environment of active drug (21).

Cytokines:

Results demonstrated that tumors such as the bladder and prostate were not immunogenic, that is the administration of irradiated tumor cells to the animal prior to subsequent administration of non-irradiated tumor cells did not result in a reduction of either the number of tumor cells to produce a tumor nor did it reduce the growth rate of the tumor. But if the tumor

was transfected with a retrovirus and secreted large concentrations of cytokines such as Il-2 then this could act as an antitumor vaccine and could also reduce the growth potential of an already established and growing tumor. IL-2 was the best, GM-CSF also had activity whereas a number of other cytokines were much less active. In clinical studies just using IL-2 for immunostimulation, very large concentrations had to be given which proved to be toxic. The key to the success of the cytokine gene modified tumor cell is that the cytokine is produced at the tumor site locally and is not toxic and that it stimulates immune recognition of the tumor and allows specific and non toxic recognition and destruction of the tumor. The exact mechanisms of how IL-2 production by the tumor cell activates immune recognition is not fully understood, but one explanation is that it bypasses the need for cytokine production by helper T cells and directly stimulates tumor antigen activated cytotoxic CD8 cells. Activation of antigen presenting cells may also occur.

<u>Tissue Promotor-Specific Chimera DNA Activation</u>

Non-Prostatic Tumor Systems:

It has been observed in non-prostatic tumors that the use of promotor specific activation can selectively lead to tissue specific gene expression of the transfected gene. In melanoma the use of the tyrosinase promotor which codes for the enzyme responsible for melanin expression produced over a 50 fold greater expression of the promotor driven reporter gene expression in melanoma cells and not non melanoma cells. Similar specific activation was seen in the melanoma cells transfected when they were growing in mice. In that experiment no non-melanoma or melanocyte cell expressed the tyrosinase drive reporter gene product. The research group at Welcome Laboratories

have cloned and sequenced the promoter region of the gene coding for carcinoembryonic antigen (CEA). CEA is expressed on colon and colon carcinoma cells but specifically on metastatic. A gene chimera was generated which cytosine deaminase. Cytosine deaminase which converts 5 flurorocytosine into 5 fluorouracil and observed a large increase in the ability to selectively kill CEA promotor driven colon tumor cells but not normal liver cells. In vivo they observed that bystander tumor cells which were not transfected with the cytosine deaminase gene were also killed, and that there was no toxicity to the host animal as the large tumors were regressing following treatment. Herpes simplex virus, (HSV), thymidine kinase similarly activates the prodrug gancyclovir to be toxic towards dividing cancer cells and HSV thymidine kinase has been shown to be specifically activatable by tissue specific promoters.

Prostatic Tumor Systems: The therapeutic key to effective cancer therapy is to achieve specificity and spare the patient toxicity. Gene therapy may provide a key part to specificity in that non-essential tissues such as the prostate and prostatic tumors produce tissue specific proteins, such as acid phosphatase (PAP), prostate specific antigen (PSA), and a gene which was cloned, prostate-specific membrane antigen (PSM). Tissues such as the prostate contain selected tissue specific transcription factors which are responsible for binding to the promoter region of the DNA of these tissue specific mRNA. The promoter for PSA has been cloned. Usually patients who are being treated for metastatic prostatic cancer have been put on androgen deprivation therapy which dramatically reduces the expression of mRNA for PSA. PSM on the other hand increases in expression with hormone deprivation which-means it would be even more intensely

expressed on patients being treated with hormone therapy.

References of Example 2

1. Coffey, D.S. Prostate Cancer - An overview of an increasing dilemma. Cancer Supplement, *71,3:* 880-886, 1993.

2. Chiarodo, A. National Cancer Institute roundtable on prostate cancer; future research directions. Cancer Res., *51:* 2498-2505, 1991.

3. Israeli, R.S., Powell, C.T., Fair, W.R., and Heston, W.D.W. Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen. Cancer Res., *53:* 227-230, 1993.

4. Horoszewicz, J.S., Kawinski, E., and Murphy, G.P. Monoclonal antibodies to a new antigenic marker in epithelial cells and serum of prostatic cancer patients. Anticancer Res., *7:* 927-936, 1987.

5. Horoszewicz, J.S., Leong, S.S., Kawinski, E., Karr, J.P., Rosenthal, H., Chu, T.M., Mirand, E.A., and Murphy, G.P. LNCaP model of human prostatic carcinoma. Cancer Res., *43:* 1809-1818, 1983.

6. Abdel-Nabi, H., Wright, G.L., Gulfo, J.V., Petrylak, D.P., Neal, C.E., Texter, J.E., Begun, F.P., Tyson, I., Heal, A., Mitchell, E., Purnell, G., and Harwood, S.J. Monoclonal antibodies and radioimmunoconjugates in the diagnosis and treatment of prostate cancer. Semin. Urol., *10:* 45-54, 1992.

7. Stone, K.R., Mickey, D.D., Wunderli, H., Mickey, G.H., and Paulson, D.F. Isolation of a human

prostate carcinoma cell line (DU-145). Int. J. Cancer, *21:* 274-281, 1978.

8. Kaign, M.E., Narayan, K.S., Ohnuki, Y., and Lechner, J.F. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). Invest. Urol., *17:* 16-23, 1979.

9. Hsu, S.M., Raine, L., and Fanger, H. Review of present methods of immunohistochemical detection. Am. J. Clin. Path. *75:* 734-738, 1981.

10. Harlow, E., and Lane, D. Antibodies: A Laboratory Manual. New York: Cold Spring Harbor Laboratory, p. 449, 1988.

11. Glisin, V., Crkvenjakov, R., and Byus, C. Ribonucleic acid isolated by cesium chloride centrifugation. Biochemistry, *13:* 2633-2637, 1974.

12. Aviv, H., and Leder, P. Purification of biologically active globin messenger RNA by chromotography on oligo-thymidylic acid cellulose. Proc. Natl. Acad. Sci. USA, *69:* 1408-1412, 1972.

13. Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T.A., Zinn, K., and Careen, M.R. Efficient in-vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucl. Acids. Res. *12:* 7035-7056, 1984.

14.

15. Axelrod, H.R., Gilman, S.C., D'Aleo, C.J., Petrylak, D., Reuter, V., Gulfo, J.V., Saad, A., Cordon-Cardo, C., and Scher, H.I. Preclinical results and human immunohistochemical studies with ^{90}Y-CYT-356; a new prostatic cancer therapeutic agent. AUA Proceedings, Abstract 596, 1992.

16. Lopes, A.D., Davis, W.L., Rosenstraus, M.J., Uveges, A.J., and Gilman, S.C. Immunohistochemical and pharmacokinetic characterization of the site-specific immunoconjugate CYT-356 derived from antiprostate monoclonal antibody 7E11-C5. Cancer Res., *50:* 6423-6429, 1990.

17. Troyer, J.K., Qi, F., Beckett, M.L., Morningstar, M.M., and Wright, G.L. Molecular characterization of the 7E11-C5 prostate tumor-associated antigen. AUA Proceedings. Abstract 482, 1993.

18. Roemer, K., Friedmann, T. Concepts and strategies for human gene therapy. FEBS. 223:212-225.

19. Antonie, P. Springer, C.J., Bagshawe, F., Searle, F., Melton, R.G., Rogers, G.T., Burke, P.J., Sherwood, R.F. Disposition of the prodrug 4-bis(2chloroethyl) amino) benzoyl-1-glutamic acid and its active parent drug in mice. Br.J.Cancer 62:909-914, 1990.

20. Connor, J. Bannerji, R., Saito, S., Heston, W.D.W., Fair, W.R., Gilboa, E. Regression of bladder tumors in mice treated with interleukin 2 gene-modified tumor cells. J.Exp.Med. 177:1127-1134, 1993. (appendix)

21. Vile R., Hart, I.R. In vitro and in vivo targeting of gene expression to melanoma cells. Cancer Res. 53:962-967, 1993.

22. Warner, J.A., Heston, W.D.W. Future developments of nonhormonal systemic therapy for prostatic carcinoma. Urologic Clinics of North America 18:25-33, 1991.

23. Vile, R.G., Hart, I.R. Use of tissue specific expression of the herpes simplex virus thymidine kinase gene to inhibit growth of established murine melanomas following direct intratumoral injection of DNA. Cancer Res. 53:3860-3864, 1993.

EXAMPLE 3:

Sensitive Detection of Prostatic Hematogenous Micrometastases Using PSA and PSM-Derived Primers in the Polymerase Chain Reaction

A PCR-based assay was developed enabling sensitive detection of hematogenous micrometastases in patients with prostate cancer. "Nested PCR", was performed by amplifying mRNA sequences unique to prostate-specific antigen and to the prostate-specific membrane antigen, and have compared their respective results. Micrometastases were detected in 2/30 patients (6.7%) by PCR with PSA-derived primers, while PSM-derived primers detected tumor cells in 19/16 patients (63.3%). All 8 negative controls were negative with both PSA and PSM PCR. Assays were repeated to confirm results, and PCR products were verified by DNA sequencing and Southern analysis. Patients harboring circulating prostatic tumor cells as detected by PSM, and not by PSA-PCR included 4 patients previously treated with radical prostatectomy and with non-measurable serum PSA levels at the time of this assay. The significance of these findings with respect to future disease recurrence and progression will be investigated.

Improvement in the overall survival of patients with prostate cancer will depend upon earlier diagnosis. Localized disease, without evidence of extra-prostatic spread, is successfully treated with either radical prostatectomy or external beam radiation, with excellent long-term results (2,3). The major problem is that approximately two-thirds of men diagnosed with prostate cancer already have evidence of advanced extra-prostatic spread at the time of diagnosis, for which there is at present no cure (4). The use of clinical serum markers such as prostate-specific

antigen (PSA) and prostatic acid phosphatase (PAP) have enabled clinicians to detect prostatic carcinomas earlier and provide useful parameters to follow responses to therapy (5). Yet, despite the advent of sensitive serum PSA assays, radionuclide bone scans, CT scans and other imaging modalities, results have not detected the presence of micrometastatic cells prior to their establishment of solid metastases. Previous work has been done utilizing the polymerase chain reaction to amplify mRNA sequences unique to breast, leukemia, and other malignant cells in the circulation and enable early detection of micrometastases (6,7). Recently, a PCR-based approach utilizing primers derived from the PSA DNA sequence was published (8). In this study 3/12 patients with advanced, stage D prostate cancer had detectable hematogenous micrometastases.

PSM appears to be an integral membrane glycoprotein which is very highly expressed in prostatic tumors and metastases and is almost entirely prostate-specific (10). Many anaplastic tumors and bone metastases have variable and at times no detectable expression of PSA, whereas these lesions appear to consistently express high levels of PSM. Prostatic tumor cells that escape from the prostate gland and enter the circulation are likely to have the potential to form metastases and are possibly the more aggressive and possibly anaplastic cells, a population of cells that may not express high levels of PSA, but may retain high expression of PSM. DNA primers derived from the sequences of both PSA and PSM in a PCR assay were used to detect micrometastatic cells in the peripheral circulation. Despite the high level of amplification and sensitivity of conventional RNA PCR, "Nested" PCR approach in which a amplified target sequence was employed, and subsequently use this PCR product as the template for another round of PCR amplification with a new set of primers totally

contained within the sequence of the previous product. This approach has enabled us to increase the level of detection from one prostatic tumor cell per 10,000 cells to better than one cell per ten million cells.

Materials and Methods

Cells and Reagents: LNCaP and MCF-7 cells were obtained from the American Type Culture Collection (Rockville, MD.). Details regarding the establishment and characteristics of these cell lines have been previously published (11,12). Cells were grown in RPMI 1640 media supplemented with L-glutamine, nonessential amino acids, obtained from the MSKCC Media Preparation Facility, and 5% fetal calf serum (Gibco-BRL, Gaithersburg, MD.) in a CO_2 incubator at 37C. All cell media was obtained from the MSKCC Media Preparation Facility. Routine chemical reagents were of the highest grade possible and were obtained from Sigma Chemical Company, St. Louis, MO.

Patient Blood Specimens: All blood specimens used in this study were from patients seen in the outpatient offices of urologists on staff at MSKCC. Two anti-coagulated (purple top) tubes per patient were obtained at the time of their regularly scheduled blood draws. Specimen procurement was conducted as per the approval of the MSKCC Institutional Review Board. Samples were promptly brought to the laboratory for immediate processing. Serum PSA and PAP determinations were performed by standard techniques by the MSKCC Clinical Chemistry Laboratory. PSA determinations were performed using the Tandem PSA assay (Hybritech, San Diego, CA.). The eight blood specimens used as negative controls were from 2 males with normal serum PSA values and biopsy-proven BPH, one healthy female, 3 healthy males, one patient with bladder cancer, and

one patient with acute promyelocytic leukemia.

Blood Sample Processing/RNA Extraction: 4 ml of whole anticoagulated venous blood was mixed with 3 ml of ice cold phosphate buffered saline and then carefully layered atop 8 ml of Ficoll (Pharmacia, Uppsala, Sweden) in a 15-ml polystyrene tube. Tubes were centrifuged at 200 x g for 30 min. at 4C. Using a sterile pasteur pipette, the buffy coat layer (approx. 1 ml.) was carefully removed and rediluted up to 50 ml with ice cold phosphate buffered saline in a 50 ml polypropylene tube. This tube was then centrifuged at 2000 x g for 30 min at 4C. The supernatant was carefully decanted and the pellet was allowed to drip dry. One ml of RNazol B was then added to the pellet and total RNA was isolated as per manufacturers directions (Cinna/Biotecx, Houston, TX.). RNA concentrations and purity were determined by UV spectroscopy on a Beckman DU 640 spectrophotometer and by gel analysis.

Determination of PCR Sensitivity: RNA was isolated from LNCaP cells and from mixtures of LNCaP and MCF-7 cells at fixed ratios (i.e. 1:100, 1:1000, etc.) using RNAzol B. Nested PCR was then performed as described below with both PSA and PSM primers in order to determine the limit of detection for the assay. LNCaP:MCF-7 (1:100,000) cDNA was diluted with distilled water to obtain concentrations of 1:1,000,000 and 1:10,000,000. MCF-7 cells were chosen because they have been previously tested and shown not to express PSM by PCR.

Polymerase Chain Reaction: The PSA outer primers used span portions of exons 4 and 5 to yield a 486 bp PCR product and enable differentiation between cDNA and possible contaminating genomic DNA amplification. The upstream primer sequence beginning at nucleotide 494 in

PSA cDNA sequence is 5'-TACCCACTGCATCAGGAACA-3' (SEQ. ID. No.) and the downstream primer at nucleotide 960 is 5'-CCTTGAAGCACACCATTACA-3' (SEQ. ID. No.). The PSA inner upstream primer (beginning at nucleotide 559) 5'-ACACAGGCCAGGTATTTCAG-3' (SEQ. ID. No.) and the downstream primer (at nucleotide 894) 5'-GTCCAGCGTCCAGCACACAG-3' (SEQ. ID. No.) yield a 355 bp PCR product. All primers were synthesized by the MSKCC Microchemistry Core Facility. 5μg of total RNA was reverse-transcribed into cDNA in a total volume of 20μl using Superscript reverse transcriptase (Gibco-BRL) according to the manufacturers recommendations. 1μl of this cDNA served as the starting template for the outer primer PCR reaction. The 20μl PCR mix included: 0.5U Taq polymerase (Promega Corp., Madison, WI.), Promega reaction buffer, 1.5mM $MgCl_2$, 200mM dNTPs, and 1.0μM of each primer. This mix was then transferred to a Perkin Elmer 9600 DNA thermal cycler and incubated for 25 cycles. The PCR profile was as follows: 94C x 15 sec., 60C x 15 sec., and 72C for 45 sec. After 25 cycles, samples were placed on ice, and 1μl of this reaction mix served as the template for another round of PCR using the inner primers. The first set of tubes were returned to the thermal cycler for 25 additional cycles. PSM-PCR required the selection of primer pairs that also spanned an intron in order to be certain that cDNA and not genomic DNA were being amplified.

The PSM outer primers yield a 946 bp product and the inner primers a 434 bp product. The PSM outer upstream primer used was 5'-ATGGGTGTTTGGTGGTATTGACC-3' (SEQ. ID. No.) (beginning at nucleotide 1401) and the downstream primer (at nucleotide 2348) was 5'-TGCTTGGAGCATAGATGACATGC-3' (SEQ. ID. No.) The PSM inner upstream primer (at nucleotide 1581) was 5'-ACTCCTTCAAGAGCGTGGCG-3' (SEQ. ID. No.) and the downstream primer (at nucleotide 2015) was 5'-

AACACCATCCCTCCTCGAACC-3' (SEQ. ID. No.). cDNA used was the same as for the PSA assay. The 501 PCR mix included: 1U Taq Polymerase (Promega), 250M dNTPs, 10mM -mercaptoethanol, 2mM $MgCl_2$, and 51 of a 10x buffer mix containing: 166mM NH_4SO_4, 670mM Tris pH 8.8, and 2 mg/ml of acetylated BSA. PCR was carried out in a Perkin Elmer 480 DNA thermal cycler with the following parameters: 94C x 4 minutes for 1 cycle, 94C x 30 sec., 58C x 1 minute, and 72C x 1 minute for 25 cycles, followed by 72C x 10 minutes. Samples were then iced and 21 of this reaction mix was used as the template for another 25 cycles with a new reaction mix containing the inner PSM primers. cDNA quality was verified by performing control reactions using primers derived from -actin yielding a 446 bp PCR product. The upstream primer used was 5'-AGGCCAACCGCGAGAAGATGA-3' (SEQ. ID. No.) (exon 3) and the downstream primer was 5'-ATGTCACACTGGGGAAGC-3' (SEQ. ID. No.) (exon 4). The entire PSA mix and 101 of each PSM reaction mix were run on 1.5-2% agarose gels, stained with ethidium bromide and photographed in an Eagle Eye Video Imaging System (Stratagene, Torrey Pines, CA.). Assays were repeated at least 3 times to verify results.

Cloning and Sequencing of PCR Products: PCR products were cloned into the pCR II plasmid vector using the TA cloning system (Invitrogen). These plasmids were transformed into competent E. coli cells using standard methods (13) and plasmid DNA was isolated using Magic Minipreps (Promega) and screened by restriction analysis. TA clones were then sequenced by the dideoxy method (14) using Sequenase (U.S. Biochemical). 3-4g of each plasmid was denatured with NaOH and ethanol precipitated. Labeling reactions were carried out according to the manufacturers recommendations using ^{35}S-dATP (NEN), and the reactions were terminated as discussed in the same protocol. Sequencing products

were then analyzed on 6% polyacrilamide/7M urea gels run at 120 watts for 2 hours. Gels were fixed for 20 minutes in 10% methanol/10% acetic acid, transferred to Whatman 3MM paper and dried down in a vacuum dryer for 2 hours at 80C. Gels were then autoradiographed at room temperature for 18 hours.

Southern Analysis: Ethidium-stained agarose gels of PCR products were soaked for 15 minutes in 0.2N HCl, followed by 30 minutes each in 0.5N NaOH/1.5M NaCl and 0.1M Tris pH 7.5/1.5M NaCl. Gels were then equilibrated for 10 minutes in 10x SSC (1.5M NaCl/0.15M Sodium Citrate. DNA was transferred onto Nytran nylon membranes (Schleicher and Schuell) by pressure blotting in 10x SSC with a Posi-blotter (Stratagene). DNA was cross-linked to the membrane using a UV Stratalinker (Stratagene). Blots were pre-hybridized at 65C for 2 hourthes and subsequently hybridized with denatured ^{32}P-labeled, random-primed cDNA probes (either PSM or PSA)(9,15). Blots were washed twice in 1x SSPE/0.5% SDS at 42C and twice in 0.1x SSPE/0.5% SDS at 50C for 20 minutes each. Membranes were air-dried and autoradiographed for 30 minutes to 1 hour at -70C with Kodak X-Omat film.

Experimental Results

PCR amplification with nested primers improved the level of detection of prostatic cells from approximately one prostatic cell per 10,000 MCF-7 cells to better than one cell per million MCF-7 cells, using either PSA or PSM-derived primers (Figures 26 and 27). This represents a substantial improvement in the ability to detect minimal disease. Characteristics of the 16 patients analyzed with respect to their clinical stage, treatment, serum PSA and PAP values, and results of the assay are shown. In total, PSA-PCR detected

tumor cells in 2/30 patients (6.7%), whereas PSM-PCR detected cells in 19/30 patients (63.3%). There were no patients positive for tumor cells by PSA and not by PSM, while PSM provided 8 positive patients not detected by PSA. Patients 10 and 11 in table 1, both with very advanced hormone-refractory disease were detected by both PSA and PSM. Both of these patients have died since the time these samples were obtained. Patients 4, 7, and 12, all of whom were treated with radical prostatectomies for clinically localized disease, and all of whom have non-measurable serum PSA values 1-2 years postoperatively were positive for circulating prostatic tumor cells by PSM-PCR, but negative by PSA-PCR. A representative ethidium stained gel photograph for PSM-PCR is shown in Figure 28. Samples run in lane A represent PCR products generated from the outer primers and samples in lanes labeled B are products of inner primer pairs. The corresponding PSM Southern blot autoradiograph is shown in Figure 29. The sensitivity of the Southern blot analysis exceeded that of ethidium staining, as can be seen in several samples where the outer product is not visible on Figure 28, but is detectable by Southern blotting as shown in Figure 29. In addition, sample 3 on Figures 28 and 29 (patient 6 in Figure 30) appears to contain both outer and inner bands that are smaller than the corresponding bands in the other patients. DNA sequencing has confirmed that the nucleotide sequence of these bands matches that of PSM, with the exception of a small deletion. This may represent either an artifact of PCR, alternative splicing of PSM mRNA in this patient, or a PSM mutation. All samples sequenced and analyzed by Southern analysis have been confirmed as true positives for PSA and PSM.

Experimental Details

The ability to accurately stage patients with prostate

cancer at the time of diagnosis is clearly of paramount importance in selecting appropriate therapy and in predicting long-term response to treatment, and potential cure. Pre-surgical staging presently consists of physical examination, serum PSA and PAP determinations, and numerous imaging modalities including transrectal ultrasonography, CT scanning, radionuclide bone scans, and even MRI scanning. No present modality, however, addresses the issue of hematogenous micrometastatic disease and the potential negative impact on prognosis that this may produce. Previous work has shown that only a fractional percentage of circulating tumor cells will inevitably go on to form a solid metastasis (16), however, the detection of and potential quantification of circulating tumor cell burden may prove valuable in more accurately staging disease. The long-term impact of hematogenous micrometastatic disease must be studied by comparing the clinical courses of patients found to have these cells in their circulation with patients of similar stage and treatment who test negatively.

The significantly higher level of detection of tumor cells with PSM as compared to PSA is not surprising to us, since more consistent expression of PSM in prostate carcinomas of all stages and grades as compared to variable expression of PSA in more poorly differentiated and anaplastic prostate cancers is noted. The detection of tumor cells in the three patients that had undergone radical prostatectomies with subsequent undetectable amounts of serum PSA was suprising. These patients would be considered to be surgical "cures" by standard criteria, yet they apparently continue to harbor prostatic tumor cells. It will be interesting to follow the clinical course of these patients as compared to others without PCR evidence of residual disease.

References of Example 3

1. Boring, C.C., Squires, T.S., and Tong, T.: Cancer Statistics, 1993. CA Cancer J. Clin., 43:7-26, 1993.

2. Lepor, H., and Walsh, P.C.: Long-term results of radical prostatectomy in clinically localized prostate cancer: Experience at the Johns Hopkins Hospital. NCI Monogr., 7:117-122, 1988.

3. Bagshaw, M.A., Cox, R.S., and Ray, G.R.: Status of radiation treatment of prostate cancer at Stanford University. NCI Monogr., 7:47-60, 1988.

4. Thompson, I.M., Rounder, J.B., Teague, J.L., et al.: Impact of routine screening for adenocarcinoma of the prostate on stage distribution. J. Urol., 137:424-426, 1987.

5. Chiarodo, A.: A National Cancer Institute roundtable on prostate cancer; future research directions. Cancer Res., 51:2498-2505, 1991.

6. Wu, A., Ben-Ezra, J., and Colombero, A.: Detection of micrometastasis in breast cancer by the polymerase chain reaction. Lab. Invest., 62:109A, 1990.

7. Fey, M.F., Kulozik, A.E., and Hansen-Hagge, T.E.: The polymerase chain reaction: A new tool for the detection of minimal residual disease in hematological malignancies. Eur. J. Cancer, 27:89-94, 1991.

8. Moreno, J.G., Croce, C.M., Fischer, R., Monne, M., Vihko, P., Mulholland, S.G., and Gomella,

L.G.: Detection of hematogenous micrometastasis in patients with prostate cancer. Cancer Res., 52:6110-6112, 1992.

9. Israeli, R.S., Powell, C.T., Fair, W.R., and Heston, W.D.W.: Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen. Cancer Res., 53:227-230, 1993.

10. Israeli, R.S., Powell, C.T., Corr, J.G., Fair, W.R., and Heston, W.D.W.: Expression of the prostate-specific membrane antigen (PSM).: Submitted to Cancer Research.

11. Horoszewicz, J.S., Leong, S.S., Kawinski, E., Karr, J.P., Rosenthal, H., Chu, T.M., Mirand, E.A., and Murphy, G.P.: LNCaP model of human prostatic carcinoma. Cancer Res., 43:1809-1818, 1983.

12. Soule, H.D., Vazquez, J., Long, A., Albert, S., and Brennan, M.: A human cell line from a pleural effusion derived from a breast carcinoma. J. Natl. Can. Inst., 51:1409-1416, 1973.

13. Hanahan, D.: Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol., 166:557-580, 1983.

14. Sanger, F., Nicklen, S., and Coulson, A.R.: DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA, 74:5463-5467, 1977.

15. Lundwall, A., and Lilja, H.: Molecular cloning of a human prostate specific antigen cDNA. FEBS Letters, 214:317, 1987.

16. Liotta, L.A., Kleinerman, J., and Saidel, G.M.: Quantitative relationships of intravascular tumor cells, tumor vessels, and pulmonary metastases following tumor implantation. Cancer Res., 34:997-1003, 1974.

EXAMPLE 4:

EXPRESSION OF THE PROSTATE SPECIFIC MEMBRANE ANTIGEN (PSM) DIMINISHES THE MITOGENIC STIMULATION OF AGGRESSIVE HUMAN PROSTATIC CARCINOMA CELLS BY TRANSFERRIN

An association between transferrin and human prostate cancer has been suggested by several investigators. It has been shown that the expressed prostatic secretions of patients with prostate cancer are enriched with respect to their content of transferrin and that prostate cancer cells are rich in transferrin receptors (J. Urol. 143, 381, 1990). Transferrin derived from bone marrow has been shown to selectively stimulate the growth of aggressive prostate cancer cells (PNAS 89, 6197, 1992). DNA sequence analysis has revealed that a portion of the coding region, from nucleotide 1250 to 1700 possesses a 54% homology to the human transferrin receptor. PC-3 cells do not express PSM mRNA or protein and exhibit increased cell growth in response to transferrin, whereas, LNCaP prostate cancer cells which highly express PSM have a very weak response to transferrin. To determine whether PSM expression by prostatic cancer cells impacts upon their mitogenic response to transferrin the full-length PSM cDNA was transfected into the PC-3 prostate cancer cells. Clones highly expressing PSM mRNA were identified by Northern analysis and expression of PSM protein was verified by Western analysis using the anti-PSM monoclonal antibody 7E11-C5.3.

2×10^4 PC-3 or PSM-transfected PC-3 cells per well ere plated in RPMI medium supplemented with 10% fetal bovine serum and at 24 hrs. added 1 μg per ml. of holotransferrin to the cells. Cells were counted at 1 day to be highly mitogenic to the PC-3 cells. Cells

were counted at 1 day to determine plating efficiency and at 5 days to determine the effect of the transferrin. Experiments were repeated to verify the results.

PC-3 cells experienced an average increase of 275% over controls, whereas the LNCaP cells were only stimulated 43%. Growth kinetics revealed that the PSM-transfected PC-3 cells grew 30% slower than native PC-3 cells. This data suggests that PSM expression in aggressive, metastatic human prostate cancer cells significantly abrogates their mitogenic response to transferrin.

The use of therapeutic vaccines consisting of cytokine-secreting tumor cell preparations for the treatment of established prostate cancer was investigated in the Dunning R3327-MatLyLu rat prostatic adenocarcinoma model. Only IL-2 secreting, irradiated tumor cell preparations were capable of curing animals from subcutaneously established tumors, and engendered immunological memory that protected the animals from another tumor challenge. Immunotherapy was less effective when tumors were induced orthotopically, but nevertheless led to improved outcome, significantly delaying, and occasionally preventing recurrence of tumors after resection of the cancerous prostate. Induction of a potent immune response in tumor bearing animals against the nonimmunogenic MatLyLu tumor supports the view that active immunotherapy of prostate cancer may have therapeutic benefits.

EXAMPLE 5:

CLONING AND CHARACTERIZATION OF THE PROSTATE SPECIFIC MEMBRANE ANTIGEN (PSM) PROMOTER.

The expression and regulation of the PSM gene is complex. By immunostaining, PSM antigen was found to be expressed brilliantly in metastasized tumor, and in organ confined tumor, less so in normal prostatic tissue and more heterogenous in BPH. PSM is strongly expressed in both anaplastic and hormone refractory tumors. PSM mRNA has been shown to be down regulated by androgen. Expression of PSM RNA is also modulated by a host of cytokines and growth factors. Knowledge of the regulation of PSM expression should aid in such diagnostic and therapeutic strategies as imunoscintigraphic imaging of prostate cancer and protate-specific promoter-driven gene therapy.

Sequencing of a 3 kb genomic DNA clone that contained 2.5 kb upstream of the transcription start site revealed that two stretches of about 300 b.p. (-260 to -600; and -1325 to -1625) have substantial homology (79-87%) to known genes. The promoter lacks a GC rich region, nor does it have a consensus TATA box. However, it contains a TA-rich region from position -35 to -65.

Several consensus recognition sites for general transcription factors such as AP1, AP2, NFkB, GRE and E2-RE were identified. Chimeric constructs containing fragments of the upstream region of the PSM gene fused to a promoterless chloramphenicol acetyl transferase gene were transfected into, and transiently expressed in LNCaP, PC-3, and SW620 (a colonic cell line). With an additional SV40 enhancer, sequence from -565 to +76

exhibited promoter activity in LNCaP but not in PC-3 nor in SW620.

Materials and Methods

Cell Lines. LNCaP and PC-3 prostatic carcinoma cell lines (American Type Culture Collection) were cultured in RPMI and MEM respectively, supplemented with 5% fetal calf serum at 37°C and 5% CO_2. SW620, a colonic cell line, is a gift from Melisa.

Polymerase Chain Reaction. The reaction was performed in a 50 μl volume with a final concentration of the following reagents: 16.6 mM NH_4SO_4, 67 mM Tris-HCl pH 8.8, acetylated BSA 0.2 mg/ml, 2mM $MgCl_2$, 250μM dNTPs, 10 mM ß-mercaptoethanol, and 1 U of rth 111 Taq polymerase (Boehringer Mannhiem, CA). A total of 25 cycles were completed with the following profile: cycle 1, 94°C 4 min.; cycle 2 through 25, 94°C 1 min, 60°C 1 min, 72°C 1 min. The final reaction was extended for 10 min at 72°C. Aliquots of the reaction were electrophoresed on 1 % agarose gels in 1X Tris-acetate-EDTA buffer.

Cloning of PSM promoter. A bacteriophage P1 library of human fibroblast genomic DNA (Genomic Sysytems, Inc., St. Louis, MI), was screened using a PCR method of Pierce et al. Primers located at the 5' end of PSM cDNA were used:5'-CTCAAAAGGGGCCGGATTTCC-3' and 5'CTCTCAATCTCACTAATGCCTC-3'. A positive clone, p683, was digested with Xho1 restriction enzyme. Southern analysis of the restricted fragments using a DNA probe from the extreme 5' to the Ava-1 site of PSM cDNA confirmed that a 3Kb fragment contains the 5' regulatory sequence of the PSM gene. The 3 kb Xho1 fragment was subcloned into pKSBluescrpt vectors and

sequenced using the dideoxy method.

Functional Assay of PSM Promoter. Chloramphenicol Acetyl Transferase, (CAT) gene plasmids were constructed from the Smal-HindIII fragments or subfragements (using either restriction enzyme subfragments or PCR) by insertion into promoterless pCAT basic or pCAT-enhancer vectors (Promega). pCAT-constructs were cotransfected with pSVßgal plasmid (5 μg of each plasmid) into cell lines in duplicates, using a calcium phosphate method (Gibco-BRL, Gaithersburg, MD). The transfected cells were harvested 72 hours later and assayed (15μg of lysate) for CAT activity using the LSC method and for ßgal activity (Promega). CAT activities were standardized by comparision to that of the ßgal activities.

Results

Sequence of the 5' end of the PSM gene.
The DNA sequence of the 3 kb XhoI fragment of p683 which includes 500 bp of DNA from the RNA start site was determined (Figures 31A-31D) Sequence 683XFRVS starts from the 5' distal end of PSM promoter, it overlaps with the published PSM putative promoter at nt 2485, i.e. the putative transcription start site is at nt 2485; sequence 683XF107 is the reverse, complement of 683XFRVS). The sequence from the XhoI fragment displayed a remarkable arrays of elements and motifs which are characteristic of eukaryotic promoters and regulatory regions found in other genes (Figure 32).

Functional Analysis of upstream PSM genomic elements for promoter activity.

Various pCAT-PSM promoter constructs were tested for promoter activities in two prostatic cell lines:

LNCaP, PC-3 and a colonic SW620 (Figure 33). Induction of CAT activity was neither observed in p1070-CAT which contained a 1070 bp PSM 5' promoter fragment, nor in p676-CAT which contained a 641 bp PSM 5' promoter fragment. However, with an additional SV-40 enhancer, sequence from -565 to +76 (p676-CATE) exhibited promoter activity in LNCaP but not in PC-3 nor in SW620.

Therefore, a LNCaP specific promoter fragment from -565 to +76 has been isolated which can be used in PSM promoter-driven gene therapy.

EXAMPLE 6:

ALTERNATIVELY SPLICED VARIANTS OF PROSTATE SPECIFIC MEMBRANE ANTIGEN RNA: RATIO OF EXPRESSION AS A POTENTIAL MEASUREMENT OF PROGRESSION

MATERIALS AND METHODS

Cell Lines. LNCaP and PC-3 prostatic carcinoma cell lines were cultured in RPMI and MEM respectively, supplemented with 5% fetal calf serum at 37°C and 5% CO_2.

Primary tissues. Primary prostatic tissues were obtained from MSKCC's in-house tumor procurement service. Gross specimen were pathologically staged by MSKCC's pathology service.

RNA Isolation. Total RNA was isolated by a modified guanidinium thiocynate/phenol/chloroform method using a RNAzol B kit (Tel-Test, Friendswood, TX). RNA was stored in diethyl pyrocarbonate-treated water at -80°C. RNA was quantified using spectrophotometric absorption at 260nm.

cDNA synthesis. Two different batches of normal prostate mRNAs obtained from trauma-dead males (Clontech, Palo Alto, CA) were denatured at 70°C for 10 min., then reverse transcribed into cDNA using random hexamers and Superscript II reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) at 50°C for 30 min. followed by a 94°C incubation for 5 min.

Polymerase Chain Reaction. Oligonucleotide primers(5'-CTCAAAAGGGGCCGGATTTCC-3' and 5'-AGGCTACTTCACTCAAAG-3'), specific for the 5' and 3' ends of PSM cDNA were designed to span the cDNA sequence. The reaction was performed in a 50 μl volume with a final concentration of the following reagents: 16.6 mM NH_4SO_4, 67 mM Tris-HCl pH 8.8, acetylated BSA 0.2 mg/ml, 2mM $MgCl_2$, 250μM dNTPs, 10 mM ß-mercaptoethanol, and 1 U of rTth polymerase (Perkin Elmer, Norwalk, CT). A total of 25 cycles were completed with the following profile: cycle 1, 94°C 4 min.; cycle 2 through 25, 94°C 1 min, 60°C 1 min, 72°C 1 min. The final reaction was extended for 10 min at 72°C. Aliquots of the reaction were electrophoresed on 1 % agarose gels in 1X Tris-acetate-EDTA buffer.

Cloning of PCR products. PCR products were cloned by the TA cloning method into pCRII vector using a kit from Invitrogen (San Diego, CA). Ligation mixture were transformed into competent *Escherichia coli* Inv5α.

Sequencing. Sequencing was done by the dideoxy method using a sequenase kit from US Biochemical (Cleveland, OH). Sequencing products were electrophoresed on a 5% polyacrylamide/7M urea gel at 52°C.

RNase Protection Assays. Full length PSM cDNA clone was digested with NgoM 1 and Nhe1. A 350 b.p. fragment

was isolated and subcloned into pSPORT1 vector (GIBCO-BRL, Gaithersburg, MD). The resultant plasmid, pSP350, was linearized, and the insert was transcribed by SP6 RNA polymerase to yield antisense probe of 395 nucleotide long, of which 355 nucleotides and/or 210 nucleotides should be protected from RNAse digestion by PSM or PSM' RNA respectively (Fig.2). Total celluar RNA (20 μg) from different tissues were hybridized to the aforementioned antisense RNA probe. Assays were performed as described (7). tRNA was used as negative control. RPAs for LNCaP and PC-3 were repeated.

RESULTS

RT-PCR of mRNA from normal prostatic tissue. Two independent RT-PCR of mRNA from normal prostates were performed as described in *Materials and Methods*. Subsequent cloning and sequencing of the PCR products revealed the presence of an alternatively spliced variant, PSM'. PSM' has a shorter cDNA (2387 nucleotides) than PSM (2653 nucleotides). The results of the sequence analysis are shown in Figure 34. The cDNAs are identical except for a 266 nucleotide region near the 5' end of PSM cDNA (nucleotide 114 to 380) that is absent in PSM' cDNA. Two independent repetitions of RT-PCR of different mRNA samples yielded identical results.

RNase Protection Assays. An RNA probe complementary to PSM RNA and spanning the 3' splice junction of PSM' RNA was used to measure relative expression of PSM and PSM' mRNAs (Figure 35). With this probe, both PSM and PSM' RNAs in LNCaP cells was detected and the predominant form was PSM. Neither PSM nor PSM' RNA was detected in PC-3 cells, in agreement with previous Northern and Western blot data (5,6). Figure 36 showed the presence of both splice variants in human primary prostatic tissues. In primary prostatic tumor, PSM is

the dominant form. In contrast, normal prostate expressed more PSM' than PSM. BPH samples showed about equal expression of both variants.

Tumor Index. The relative expression of PSM and PSM' (Figure 36) was quantified by densitometry and expressed as a tumor index (Figure 37). LNCaP has an index ranging from 9-11; CaP from 3-6; BPH from 0.75 to 1.6; normal prostate has values from 0.075 to 0.45.

DISCUSSION

Sequencing data of PCR products derived from human normal prostatic mRNA with 5' and 3' end PSM oligonucleotide primers revealed a second splice variant, PSM', in addition to the previously described PSM cDNA.

PSM is a 750 a.a. protein with a calculated molecular weight of 84,330. PSM was hypothesized to be a type II integral membrane protein (5). A classic type II membrane protein is the transferrin receptor and indeed PSM has a region that has modest homology with the transferrin receptor (5). Analysis of the PSM amino acid sequence by either the methods of Rao and Argos (7) or Eisenburg *et. al.* (8) strongly predicted one transmembrane helix in the region from a.a.#20 to #43. Both programs found other regions that could be membrane associated but were not considered likely candidates for being transmembrane regions.

PSM' antigen, on the other hand, is a 693 a.a. protein as deduced from its mRNA sequence with a molecular weight of 78,000. PSM' antigen lacks the first 57 amino acids present in PSM antigen (Figure 34). It is likely that PSM' antigen is cytosolic.

The function of PSM and PSM' are probably different.

The cellular location of PSM antigen suggests that it may interact with either extra- or intra- cellular ligand(s) or both; while that of PSM' implies that PSM' can only react with cytosolic ligand(s). Furthermore, PSM antigen has 3 potential phosphorylation sites on its cytosolic domain. These sites are absent in PSM' antigen. On the other hand, PSM' antigen has 25 potential phosphorylation sites, 10 N-myristoylation sites and 9 N-glycosylation sites. For PSM antigen, all of these potential sites would be on the extracellular surface. The modifications of these sites for these homologous proteins would be different depending on their cellular locations. Consequently, the function(s) of each form would depend on how they are modified.

The relative differences in expression of PSM and PSM' by RNase protection assays was analyzed. Results of expression of PSM and PSM' in primary prostatic tissues strongly suggested a relationship between the relative expression of these variants and the status of the cell: either normal or cancerous. While it is noted here that the sample size of the study is small (Figures 36 and 37), the consistency of the trend is evident. The samples used were gross specimens from patients. The results may have been even more dramatic if specimens that were pure in content of CaP, BPH or normal had been used. Nevertheless, in these specimens, it is clear that there is a relative increase of PSM over PSM' mRNA in the change from normal to CaP. The Tumor Index (Figure 37) could be useful in measuring the pathologic state of a given sample. It is also possible that the change in expression of PSM over PSM' may be a reason for tumor progression. A more differentiated tumor state may be restored by PSM' either by transfection or by the use of differentiation agents.

References of Example 6

1. Murphy, G.P. Report on the American Urologic Association/American Cancer Society Scientific Seminar on the Detection and treatment of Early-Stage Prostate Cancer. CA Cancer J. Clin. 44:91-95,1994.

2. Israeli, R.S., Miller Jr., W.H., Su, S.L., Powell, C.T., Fair, W.R., Samadi, D.S., Huryk, R.F., DelBlasio, A., Edwards, E.T, and Heston, W.D.W. Sensitive Nested Reverse Transcription Polymerase Chain Reaction Detection of Circulating Prostatic Tumor Cells: Comparision of Prostate-specific Membrane Antigen and Prostate-specific Antigen-based Assays. Cancer Res., 54: 6325-6329,1994.

3. Horoszewicz, J.S., Kawinski, E., and Murphy, G.P. Monoclonal antibodies to a new antigenic marker in epithelial cells and serum of prostatic cancer patients. Anticancer Res., 7:927-936,1987.

4. Horoszewicz, J.S., Leong, S.S., Kawinski, E., Karr, J.P., Rosenthal, H., Chu, T.M., Mirand, E.A. and Murphy, G.P. LNCaP model of human prostatic Carcinoma. Cancer Res., 43:1809-1818,1983.

5. Israeli, R.S., Powell, C.T., Fair, W.R. and Heston, W.D.W. Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen. Cancer Res.,53:227-230,1993.

6. Israeli, R.S., Powell, C.T., Corr, J.G., Fair, W.R. and Heston, W.D.W. Expression of the prostate-specific membrane antigen. Cancer Res., 54:1807-1811,1994.

7. Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res.,12:7035-7056, 1984.

8. Rao, M.J.K. and Argos, P. A conformational preference parameter to predict helices in integral membrane proteins. Biochim. Biophys. Acta, 869:197-214,1986.

9. Eisenburg, D., Schwarz, E., Komaromy, M. and Wall, R. Analysis of membrane and surface protein sequences with the hydrophbic moment plot, J. Mol. Biol. 179:125-142,1984.

10. Troyer, J.K. and Wright Jr.,G.L. Biochemical characterization and mapping of 7E-11 C-5.3. Epitope of the prostate specific membrane antigen (PSMA). American Association for Cancer Research Special Conference: Basic and Clinical Aspect of Prostate Cancer. Abstract C-38, 1994.

EXAMPLE 7:

ENHANCED DETECTION OF PROSTATIC HEMATOGENOUS MICRO-METASTASES WITH PSM PRIMERS AS COMPARED TO PSA PRIMERS USING A SENSITIVE NESTED REVERSE TRANSCRIPTASE-PCR ASSAY.

77 randomly selected samples were analyzed from patients with prostate cancer and reveals that PSM and PSA primers detected circulating prostate cells in 48 (62.3%) and 7 (9.1%) patients, respectively. In treated stage D disease patients, PSM primers detected cells in 16 of 24 (66.7%), while PSA primers detected cells in 6 of 24 patients (25%). In hormone-refractory prostate cancer (stage D3), 6 of 7 patients were positive with both PSA and PSM primers. All six of these patients died within 2-6 months of their assay, despite aggressive cytotoxic chemotherapy, in contrast to the single patient that tested negatively in this group and is alive 15 months after his assay, suggesting that PSA-PCR positivity may serve as a predictor of early mortality. In post-radical prostatectomy patients with negative serum PSA values, PSM primers detected metastases in 21 of 31 patients (67.7%), while PSA primers detected cells in only 1 of 33 (3.0%), indicating that micrometastatic spread may be a relatively early event in prostate cancer. The analysis of 40 individuals without known prostate cancer provides evidence that this assay is highly specific and suggests that PSM expression may predict the development of cancer in patients without clinically apparent prostate cancer. Using PSM primers, micrometastases were detected in 4 of 40 controls, two of whom had known BPH by prostate biopsy and were later found to have previously undetected prostate cancer following repeat prostate biopsy

performed for a rising serum PSA value. These results show the clinical significance of detection of hematogenous micrometastatic prostate cells using PSM primers and potential applications of this molecular assay.

EXAMPLE 8:

MODULATION OF PROSTATE SPECIFIC MEMBRANE ANTIGEN (PSM) EXPRESSION IN VITRO BY CYTOKINES AND GROWTH FACTORS.

The effectiveness of CYT-356 imaging is enhanced by manipulating expression of PSM. PSM mRNA expression is downregulated by steroids. This is consistent with the clinical observations that PSM is strongly expressed in both anaplastic and hormone refractory lesions. In contrast, PSA expression is decreased following hormone withdrawal. In hormone refractory disease, it is believed that tumor cells may produce both growth factors and receptors, thus establishing an autocrine loop that permits the cells to overcome normal growth constraints. Many prostate tumor epithelial cells express both TGFα and its receptor, epidermal growth factor receptor. Results indicate that the effects of TGFα and other selected growth factors and cytokines on the expression of PSM in-vitro, in the human prostatic carcinoma cell line LNCaP.

2×10^6 LNCaP cells growing in androgen-depleted media were treated for 24 to 72 hours with EGF, TGFα, TNFß or TNFα in concentrations ranging from 0.1 ng/ml to 100 ng/ml. Total RNA was extracted from the cells and PSM mRNA expression was quantitated by Northern blot analysis and laser densitometry. Both b-FGF and TGFα yielded a dose-dependent 10-fold upregulation of PSM expression, and EGF a 5-fold upregulation, compared to untreated LNCaP. In contrast, other groups have shown

a marked downregulation in PSA expression induced by these growth factors in this same in-vitro model. TNFα, which is cytotoxic to LNCaP cells, and TNFß downregulated PSM expression 8-fold in androgen depleted LNCaP cells.

TGFα is mitogenic for aggressive prostate cancer cells. There are multiple forms of PSM and only the membrane form is found in association with tumor progression. The ability to manipulate PSM expression by treatment with cytokines and growth factors may enhance the efficacy of Cytogen 356 imaging, and therapeutic targeting of prostatic metastases.

EXAMPLE 9:

NEOADJUVANT ANDROGEN-DEPRIVATION THERAPY (ADT) PRIOR TO RADICAL PROSTATECTOMY RESULTS IN A SIGNIFICANTLY DECREASED INCIDENCE OF RESIDUAL MICROMETASTATIC DISEASE AS DETECTED BY NESTED RT-PCT WITH PRIMERS.

Radical prostatectomy for clinically localized prostate cancer is considered by many the "gold standard" treatment. Advances over the past decade have served to decrease morbidity dramatically. Improvements intended to assist clinicians in better staging patients preoperatively have been developed, however the incidence of extra-prostatic spread still exceeds 50%, as reported in numerous studies. A phase III prospective randomized clinical study designed to compare the effects of ADT for 3 months in patients undergoing radical prostatectomy with similarly matched controls receiving surgery alone was conducted. The previously completed phase II study revealed a 10% margin positive rate in the ADT group (N=69) as compared to a 33% positive rate (N=72) in the surgery alone group.

Patients who have completed the phase III study were analyzed to determine if there are any differences between the two groups with respect to residual micrometastatic disease. A positive PCR result in a post-prostatectomy patient identifies viable metastatic cells in the circulation.

Nested RT-PCR was performed with PSM primers on 12 patients from the ADT group and on 10 patients from the control group. Micrometastatic cells were detected in 9/10 patients (90%) in the control group, as compared to only 2/12 (16.7%) in the ADT group. In the ADT group, 1 of 7 patients with organ-confined disease tested positively, as compared to 3 of 3 patients in the control group. In patients with extra-prostatic disease, 1 of 5 were positive in the ADT group, as compared to 6 of 7 in the control group. These results indicate that a significantly higher number of patients may be rendered tumor-free, and potentially "cured" by the use of neoadjuvant ADT.

EXAMPLE 10:

SENSITIVE NESTED RT-PCR DETECTION OF CIRCULATION PROSTATIC TUMOR CELLS - COMPARISON OF PSM AND PSA-BASED ASSAYS

Despite the improved and expanded arsenal of modalities available to clinician today, including sensitive serum PSA assays, CT scan, transrectal ultrasonography, endorectal co.I MRI, etc., many patients are still found to have metastatic disease at the time of pelvic lymph node dissection and radical prostatectomy. A highly sensitive reverse transcription PCR assay capable of detecting occult hematogenous micrometastatic prostatic cells that would otherwise go undetected by presently available staging modalities

was developed. This assay is a modification of similar PCR assays performed in patients with prostate cancer and other malignancies[2,3,4,5]. The assay employs PCR primers derived from the cDNA sequences of prostate-specific antigen[6] and the prostate-specific membrane antigen recently cloned and sequenced.

Materials and Methods

Cells and Reagents. LNCaP and MCF-7 cells were obtained from the American Type Culture Collection (Rockville, MD.). Details regarding the establishment and characteristics of these cell lines have been previously published[8,9]. Cells grown in RPMI 1640 medium and supplemented with L-glutamine, nonessential amino acids, and 5% fetal calf serum (Gibco-BRL, Gaithersburg, MD.) In a 5% CO_2 incubator at 37°C. All cell media was obtained from the MSKCC Media Preparation Facility. Routine chemical reagents were of the highest grade possible and were obtained from Sigma Chemical Company (St. Louis, MO).

Patient Blood Specimens. All blood specimens used in this study were from patients seen in the outpatient offices of urologists on staff at MSKCC. Two anti-coagulated tubes per patient were obtained at the time of their regularly scheduled blood draws. Specimens were obtained with informed consent of each patient , as per a protocol approved by the MSKCC Institutional Review Board. Samples were promptly brought to the laboratory for immediate processing. Seventy-seven specimens from patients with prostate cancer were randomly selected and delivered to the laboratory "blinded" along with samples from negative controls for processing. These included 24 patients with stage D disease (3 with D_0, 3 with D^1, 11 with D^2, and 7 with D^3), 31 patients who had previously undergone radical

prostatectomy and had undetectable postoperative serum PSA levels (18 with pT2 lesions, 11 with pT3, and 2 pT4), 2 patients with locally recurrent disease following radical prostatectomy, 4 patients who had received either external beam radiation therapy or interstitial 1^{125} implants, 10 patients with untreated clinical stage T1-T2 disease, and 6 patients with clinical stage T3 disease on anti-androgen therapy. The forty blood specimens used as negative controls were from 10 health males, 9 males with biopsy-proven BPH and elevated serum PSA levels, 7 healthy females, 4 male patients with renal cell carcinoma, 2 patients with prostatic intraepithelial neoplasia (PIN), 2 patients with transitional cell carcinoma of the bladder and a pathologically normal prostate, 1 patient with acute prostatitis, 1 patient with acute promyelocytic leukemia, 1 patient with testicular cancer, 1 female patient with renal cell carcinoma, 1 patient with lung cancer, and 1 patient with a cyst of the testicle.

Blood Sample Processing/RNA Extraction. 4 ml of whole anticoagulated venous blood was mixed with 3 ml of ice cold PBS and then carefully layered atop 8 ml of Ficoll (Pharmacia, Uppsala, Sweden) in a 14-ml polystyrene tube. Tubes were centrifuged at 200 x g for 30 min. at 4°C. The buffy coat layer (approx. 1 ml.) was carefully removed and rediluted to 50 ml with ice cold PBS in a 50 ml polypropylene tube. This tube was then centrifuged at 2000 x g for 30 min. at 4°C. The supernatant was carefully decanted and the pellet was allowed to drip dry. One ml of RNazol B was then added to the pellet and total RNA was isolated as per manufacturers directions (Cinna/Biotecx, Houston, TX.) RNA concentrations and purity were determined by UV spectroscopy on a Beckman DU 640 spectrophotometer and by gel analysis.

Determination of PCR Sensitivity. RNA was isolated from LNCaP cells and from mixtures of LNCaP and MCF-7 cells at fixed ratios (i.e. 1:100, 1:1,000, etc.) using RNAzol B. Nested PCR was then performed as described below with both PSA and PSM primers in order to determine the limit of detection for the assay. LNCaP:MCF-7 (1:100,000) cDNA was diluted with distilled water to obtain concentrations of 1:1,000,000. The human breast cancer cell line MCF-7 was chosen because they had previously been tested by us and shown not to express either PSM nor PSA by both immunohistochemistry and conventional and nested PCR.

Polymerase Chain Reaction. The PSA outer primer sequences are nucleotides 494-513 (sense) in exon 4 and nucleotides 960-979 (anti-sense) in exon 5 of the PSA cDNA. These primers yield a 486 bp PCR product from PSA CDNA that can be distinguished from a product synthesized from possible contaminating genomic DNA.

PSA-494 5'-TAC CCA CTG CAT CAG GAA CA-3'

PSA-960 5'-CCT TGA AGC ACA CCA TTA CA-3'

The PSA inner upstream primer begins at nucleotide 559 and the downstream primer at nucleotide 894 to yield a 355 bp PCR product.

PSA-559 5'-ACA CAG GCC AGG TAT TTC AG-3'

PSA-894 5'-GTC CAG CGT CCA GCA CAC AG-3'

All primers were synthesized by the MSKCC Microchemistry Core Facility. 5μg of total RNA was reverse-transcribed into cDNA using random hexamer primers (Gibco-BRL) and Superscript II reverse transcriptase (Gibco-BRL) according to the manufacturers recommendations. 1μl of this CDNA served as the starting template for the outer primer PCR reaction. The 20μl PCR mix included: 0.5U Taq polymerase (Promega) Promega reaction buffer, 1.5mM $MgCl_2$, 200μM dNTPs, and 1.0μM of each primer. This mix

was then transferred to a Perkin Elmer 9600 DNA thermal cycler and incubated for 25 cycles. The PCR profile was as follows: 94°C x 15 sec., 60°C x 15 sec., and 72°C for 45 sec. After 25 cycles, samples were placed on ice, and 1μl of this reaction mix served as the template for another 25 cycles using the inner primers. The first set of tubes were returned to the thermal cycler for 25 additional cycles. The PSM outer upstream primer sequences are nucleotides 1368-1390 and the downstream primers are nucleotides 1995-2015, yielding a 67 bp PCR product.

PSM-1368 5'-CAG ATA TGT CAT TCT GGG AGG TC-3'
PSM-2015 5'-AAC ACC ATC CCT CCT CGA ACC-3'

The PSM inner upstream primer span nucleotides 1689-1713 and the downstream primer span nucleotides 1899-1923, yielding a 234 bp PCR product.

PSM-1689 5'-CCT AAC AAA AGA GCT GAA AAG CCC-3'
PSM-1923 5'-ACT GTG ATA CAG TGG ATA GCC GCT-3'

2μl of cDNA was used as the starting DNA template in the PCR assay. The 50μl PCR mix included: 1U Taq polymerase (Boehringer Mannheim), 250μM cNTPs, 10mM ß-mercaptoethanol, 2mM $MgCl_2$, and 5μl of a 10x buffer mix containing: 166mM NH_4SO_4, 670mM Tris pH 8.8, and 2mg/ml of acetylated BSA. PCR was carried out in a Perkin Elmer 480 DNA thermal cycler with the following parameters: 94°C x 4 minutes for 1 cycle, 94°C x 30 sec., 58°C x 1 minute, and 72°C x 1 minute for 25 cycles, followed by 72°C x 10 minutes. Samples were then iced and 2.5μl of this reaction mix was used as the template for another 25 cycles with a new reaction mix containing the inner PSM primers. cDNA quality was verified by performing control reactions using primers derived from the ß-2-microglobulin gene sequence[10] a ubiquitous housekeeping gene. These primers span exons 2-4 and generate a 620 bp PCR product. The sequences for these primers are:

ß-2 (exon 2) 5'-AGC AGA GAA TGG AAA GTC AAA-3'
ß-2 (exon 4) 5'-TGT TGA TGT TGG ATA AGA GAA-3'

The entire PSA mix and 7-10μl of each PSM reaction mix were run on 1.5-2% agarose gels, stained with ethidium bromide and photographed in an Eage Eye Video Imaging System (Statagene, Torrey Pines, CA.). Assays were repeated at least twice to verify results.

Cloning and Sequencing of PCR Products. PCR products were cloned into the pCR II plasmid vector using the TA cloning system (Invitrogen). These plasmids were transformed into competent E. coli cells using standard methods[11] and plasmid DNA was isolated using Magic Minipreps (Promega) and screened by restriction analysis. Double-stranded TA clones were then sequenced by the dideoxy method[12] using ^{35}S-cCTP (NEN) and Sequenase (U.S. Biochemical). Sequencing products were then analyzed on 6% polyacrilamide/7M urea gels, which were fixed, dried, and autoradiographed as described.

Southern Analysis. PCR products were transferred from ethidium-stained agarose gels to Nytran nylon membranes (Schletcher and Schuell) by pressure blotting with a Posi-blotter (Stratagene) according to the manufacturer's instructions. DNA was cross-linked to the membrane using a UV Stratalinker (Stratagene). Blots were pre-hybridized at 65°C for 2 hours and subsequently hybridized with denatured ^{32}P-labeled, random-primed[13] cDNA probes (either PSA or PSM). Blots were washed twice in 1x SSC/0.5% SDS at 42°C and twice in 0.1x SSC/0.1% SDS at 50°C for 20 minutes each. Membranes were air-dried and autoradiographed for 1-3 hours at room temperature with Hyperfilm MP (Amersham).

Results

PSA and PSM Nested PCR Assays: The application of nested PCR increased the level of detection from an average of 1:10,000 using outer primers alone, to better than 1:1,000,000. Dilution curves demonstrating this added sensitivity are shown for PSA and PSM-PCR in Figures 1 and 2 respectively. Figure 1 shows that the 486 bp product of the PSA outer primer set is clearly detectable with ethidium staining to 1:10,000 dilutions, whereas the PSA inner primer 355 bp product is clearly detectable in all dilutions shown. In Figure 2 the PSM outer primer 647 bp product is also clearly detectable in dilutions to only 1:10,000 with conventional PCR, in contrast to the PSM inner nested PCR 234 bp product which is detected in dilutions as low as 1:1,000,000. Southern blotting was performed on all controls and most of the patient samples in order to confirm specificity. Southern blots of the respective dilution curves confirmed the primer specificities but did not reveal any significantly increased sensitivity.

PCR in Negative Controls: Nested PSA and PSM PCR was performed on 40 samples from patients and volunteers as described in the methods and materials section. Figure 48 reveals results from 4 representative negative control specimens, in addition to a positive control. Each specimen in the study was also assayed with the ß-2-microglobulin control, as shown in the figure, in order to verify RNA integrity. Negative results were obtained on 39 of these samples using the PSA primers, however PSM nested PCR yielded 4 positive results. Two of these "false positives" represented patients with elevated serum PSA values and an enlarged prostate who underwent a transrectal prostate biopsy revealing stromal and fibromuscular hyperplasia. In both of

these patients the serum PSA level continued to rise and a repeat prostate biopsy performed at a later date revealed prostate cancer. One patient who presented to the clinic with a testicular cyst was noted to have a positive PSM nested PCR result which has been unable to explain. Unfortunately, this patient never returned for follow up, and thus have not been able to obtain another blood sample to repeat this assay. Positive result were obtained with both PSA and PSM primers in a 61 year old male patient with renal cell carcinoma. This patient has a normal serum PSA level and a normal digital rectal examination. Overall, if the two patients were excluded in whom a positive PCR, but no other clinical test, accurately predicted the presence of prostate cancer, 36/38 (94.7%) of the negative controls were negative with PSM primers, and 39/40 (97.5%) were negative using PSA primers.

Patient Samples: In a "blinded" fashion, in which the laboratory staff were unaware of the nature of each specimen, 117 samples from 77 patients mixed randomly with 40 negative controls were assayed. The patient samples represented a diverse and heterogeneous group as described earlier. Several representative patient samples are displayed in Figure 49, corresponding to positive results from patients with both localized and disseminated disease. Patients 4 and 5, both with stage D prostate cancer exhibit positive results with both the outer and inner primer pairs, indicating a large circulating tumor cell burden, as compared to the other samples. Although the PSM and PSA primers yielded similar sensitivities in LNCaP dilution curves as previously shown, PSM primers detected micrometastases in 62.3% of the patient samples, whereas PSA primers only detected 9.1%. In patients with documented metastatic prostate cancer (stages D_0 - D_3) receiving anti-androgen treatment, PSM primers

detected micrometastases in 16/24 (66.7%), whereas PSA primers detected circulating cells in only 6/24 (25%). In the study 6/7 patients with hormone-refractory prostate cancer (stage D_3) were positive. In the study, PSA primers revealed micrometastatic cells in only 1/15 (6.7%) patients with either pT3 or pT4 (locally-advanced) prostate cancer following radical prostatectomy. PSM primers detected circulating cells in 9/15 (60%) of these patients. Interestingly, circulating cells 13/18 (72.2%) patients with pT2 (organ-confined) prostate cancer following radical prostatectomy using PSM primers was detected. None of these patient samples were positive by PSA-PCR.

Improved and more sensitive method for the detection of minimal, occult micrometastic disease have been reported for a number of malignancies by use of immunohistochemical methods (14), as well as the polymerase chain reaction (3, 4, 5). The application of PCR to detect occult hematogenous micrometastases in prostate cancer was first described by Moreno, et al. (2) using conventional PCR with PSA-derived primers.

When human prostate tumors and prostate cancer cells in-vitro were studied by immunohistochemistry and mRNA analysis, PSM appeared to be highly expressed in anaplastic cells, hormone-refractory cells, and bony metastases (22, 23, 24), in contrast to PSA. If cells capable of hematogenous micrometastasis represent the more aggressive and poorly-differentiated cells, they may express a higher level of PSM per cell as compared to PSA, enhancing their detectibility by RT-PCR.

Nested RT-PCR assays are both sensitive and specific. Results have been reliably reproduced on repeated occasions. Long term testing of both cDNA and RNA stability is presently underway. Both assays are

capable of detecting one prostatic cell in at least one million non-prostatic cells of similar size. This confirms the validity of the comparison of PSM vs. PSA primers. Similar levels of PSM expression in both human prostatic cancer cells in-vivo and LNCaP cells in-vitro resulted. The specificity of the PSM-PCR assay was supported by the finding that two "negative control" patients with positive PSM-PCR results were both subsequently found to have prostate cancer. This suggests an exciting potential application for this technique for use in cancer screening. In contrast to recently published data (18), significant ability for PSA primers to accurately detect micrometastatic cells in patients with pathologically with pathologically organ-confined prostate cancer, despite the sensitivity of the assay failed to result. Rather a surprisingly high percentage of patients with localized prostate cancer that harbor occult circulating prostate cells following "curative" radical prostatectomy results which suggests that micrometastasis is an early event in prostate cancer.

The application of this powerful new modality to potentially stage and/or follow the response to therapy in patients with prostate cancer certainly merits further investigation. In comparison to molecular detection of occult tumor cells, present clinical modalities for the detection of prostate cancer spread appear inadequate.

References for Example 10

1. Boring, C.C., Squires, T.S., Tong, T., and Montgomery, S. Cancer Statistics, 1994. CA., 44: 7-26, 1994.

2. Moreno, J.G., Croce, C.M., Fischer, R., Monne, M., Vihko, P., Mulholland, S.G., and Gomella, L.G., Detection of hematogenous micrometastasis in patients with prostate cancer. Cancer Res., 52:6110-6112, 1992.

3. Wu, A., Ben-Ezra, J., and Colombero, A.: Detection of micrometastasis in breast cancer by the polymerase chain reaction. Lab. Ivest., 62: 109A, 1990.

4. Fey, M.F., Kulozik, A.E., and Hansen-Hagge, T.E.: The polymerase chain reactipn: A new tool for the detection of minimal residual disease in hematological malignacies. Eur. J. Cancer, 27: 89-94, 1991.

5. Miller, W.H., Jr., Levine, K., DeBlasio, A., Frankel, S.R., Dmitrovsky, E., and Warrell, R.P., Jr. Detection of mininal residual disease in Acute Promyelocytic Leukemia by a reverse transciption polymerase chain reaction assay for th PML/RAR-α fusion mRNA. Blood, 82: 1689-1694, 1993.

6. Lundwall, A., and Lilja, H: Molecular cloning of a human prostate specific antigen cDNA. FEBS Letters, 214: 317, 1987.

7. Isaeli, R.S., Powell, C.T., Fair, W.R., and Heston, W.D.W.: Molecular cloning of a

complementary DNA encoding a prostate-specific membran antigen. Cancer Res., 53: 227-230, 1993.

8. Horoszewicz, J.S., Leong, S.S., Kawinski, E., Karr, J.P., Rosenthal, H., Chu, T.M., Mirand, E.A., and Murphy, G.P.: LNCaP model of human prostactic carcinoma. Cancer Res., 43: 1809-1818, 1983.

9. Soule, H.D., Vazquez, J., Long, A., Albert, S., and Brennan, M.: A human cell line from a pleural effusion derived from a breast carcinoma. J. Natl. Can. Inst., 51: 1409-1416, 1973.

10. Gussow, D., Rein, R., Ginjaar, I., Hochstenbach, F., Seemann, G., Kottman, A., Ploegh, H.L. The human ß-2-Microglobulin gene. Primary structure and definition of the transcriptional unit. J. of Immunol. 139:3132-3138, 1987.

11. Hanahan, D.: Studies on transformation of Escherichia coli with plasmids. J. Mol. Biol., 166:557-580, 1983.

12. Sanger, F., Nicklen, S., and Coulson, A.R.: DNA sequncing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA, 74:5463-5467, 1977.

13. Feinberg, A.P., and Vogelstein, B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem., 132:6-13, 1983.

14. Oberneder, R., Riesenberg, R., Kriegmair, M., Bitzer, U., Klammert, R., Schneede, P., Hofstetter, A., Riethmuller, G., and Pantel, K. Immunocytochemcical detection and phenytypic

characterization of micrometastatic tumour cells in bone marrow of patients with prostate cancer. Urol. Res. 22:3-8, 1994.

15. Israeli, R.S., Miller, W.H., Jr., Su, S.L., Samadi, D.S., Powell, C.T., Heston, W.D.W., Wise, G.J., and Fair, W.R. Sensitive detection of prostatic hematogenous micrometastases using prostate-specific antigen (PSA) and prostate-specific membran antigen (PSM) derived primers in the polymerase chain reaction. J. Urol. 151:373A, 1994.

16. Israeli, R.S., Miller, W.H., Jr., Su, S.L, Samadi, D.S., Powell, C.T. Heston, W.D.W., Wise, G.J., and Fair, W.S. Sensitive detection of prostatic hematogenous micrometastases using PsA and PSM-derived primers in the polymerase chain reaction. In press - J. Urology.

17. Vessella, R., Stray, J., Arman, E., Ellis, W., and Lange, P. Reverse transcription polymerase chain reaction (RT-PCR) detects metastatic prostate cancer cells in lymph nodes, blood and potentially bone marrow using PSA-mRNA as template, J. Urol. 151:412A, 1994.

18. Katz, A.E., Olsson, C.A., Raffo, A.J., Cama, C., Perlman, H., Seaman, E., O'Toole, K.M., McMahon, D., Benson, M., and Buttyan, R., Molecular staging of prostate cancer with the use of an enhanced reverse transcriptase-PCR assay. Urology 43:765-775, 1994.

19. Wood, D.P., Jr., Banks, E.R., Humphries, S., McRoberts, J.W., and Rangenkar, V.M. Identification of micrometastases in paitents

with prostate cancer. J. Urol. 151:303A, 1994.

20. Deguchi, T., Doi, T., Ehara, H., Ito, S., Takahashi, Y., Nishino, Y., Fujihiro, S., Kawamura, T., Komeda, H., Horie, M., Kaji, H., Shimokawa, K., Tanaka, T., and Kawada, Y. Detection of micrometastic prostate cancer cells in lymph nodes by reverse-transcriptase polymerase chain reaction. Cancer Res. 53:5350-4, 1993.

21. Ghossein, R., Scher, H., Gerald, W., Hoffman, A., Kelley, W., Curely, T., Libertz, C., and Rosai, J. Detection of cirulating tumor cells in peripheral blood of patients with advanced prostatic carcinoma. Proc. Amer. Soc. of Clin. Oncol., 13:237, 1994.

22. Israeli, R.S., Powel, C.T., Corr, J.G., Fair, W.R., and Heston, W.D.W.: Expression of the prostate-specific membrane antigen. Cancer Res., 54:1807-1811, 1994.

23. Axelrod, H.R., Gilman, S.C., D'Aleo, C.H. Petrylak, D., Reuter, V., Gulfo, J.V., Saad A., Cordon-Cardo, C., and Scher, H.I. Preclinical results and human immunohistochemical strudies with ^{90}Y-CYT-356: a new prostatic cancer therapeutic agent. J.Urol., 147:361A, 1992.

24. Wright, G.L., Jr., Haley, C., Beckett, M.L., and Schellhammer, P.F. Expression of the prostate biomaker 7E11-C5 in primary and metastic prostate carcinoma. Proc. Amer. Ass. for Can. Res. 35:233, 1994.

25. Liotta, L.A., Kleinerman, J., and Saidel, G.M.:

Quantitative relationships of intravascular tumor cells, tumors vessels, and pulmonary metastases following tumore implantation. Cancer Res., 34:997-1003, 1974.

EXAMPLE 11:

CHROMOSOMAL LOCALIZATION OF COSMID CLONES 194 AND 683 BY FLUORESCENCE IN-SITU HYBRIDIZATION:

PSM was initially mapped as being located on chromosome 11p11.2-p13 (Figures 51-54). Further information from the cDNA in-situ hybridizations experiments demonstrated as much hybridization on the q as p arms. Much larger fragments of genomic DNA was obtained as cosmids and two of these of about 60 kilobases each one going 3' and the other 5' both demonstrated binding to chromosome 11 p and q under low stringency. However under higher stringency conditions only the binding at 11q14-q21 remained. This result suggests that there is another gene on 11p that is very similar to PSM because it is so strongly binding to nearly 120 kilobases of genomic DNA (Figure 50).

Purified DNA from cosmid clones 194 and 683 was labelled with biotin dUTP by nick translation. Labelled probes were combined with sheared human DNA and independently hybridized to normal metaphase chromosomes derived from PHA stimulated peripheral blood lymphocytes in a solution containing 50% formamide, 10% dectran sulfate, and 2XSSC. Specific hybridization signals were detected by incubating the hybridized slides in fluoresein conjugated avidin. Following signal detection the slides were counterstained with propidium iodide and analyzed. These first experiments resulted in the specific labelling of a group C chromosome on both the long and short arms. This chromosome was believed to be chromosome 11 on the basis of its size and morphology. A second set of experiments were performed in which a chromosome 11 centromere specific probe was

cohybridized with the cosmid clones. These experiments were carried out in 60% formamide in an attempt to eliminate the cross reactive signal which was observed when low stringency hybridizations were done. These experiments resulted in the specific labelling of the centromere and the long arm of chromosome 11. Measurements of 10 specifically labelled chromosomes 11 demonstrated that the cosmid clones are located at a position which is 44% of the distance from the centromere to the telomere of chromosome arm 11q, an area that corresponds to band 14q. A total of 160 metaphase cells were examined with 153 cells exhibiting specific labelling.

Cloning of the 5' upstream and 3' downstream regions of the PSM genomic DNA. A bacteriophage P1 library of human fibroblast genomic DNA (Genomic Systems, St. Louis, MI) was screened using the PCR method of Pierce et. al. Primer pairs located at either the 5' or 3' termini of PSM cDNA were used. Positive cosmid clones were digested with restriction enzymes and confirmed by Southern analysis using probes which were constructed from either the 5' or 3' ends of PSM cDNA. Positive clone p683 contains the 5' region of PSM cDNA and about 60 kb upstream region. Clone -194 contains the 3' terminal of the PSM cDNA and about 60 kb downstream.

EXAMPLE 12:

PEPTIDASE ENZYMATIC ACTIVITY

PSM is a type two membrane protein. Most type two membrane proteins are binding proteins, transport proteins or peptidases. PSM appears to have peptidase activity. When examining LNCaP cells with a substrate N-acetyl-aspartyl-^{14}C-glutamic acid, NAAG, glutamic acid was released, thus acting as a carboxypeptidase. In

vitro translated PSM message also had this peptidase activity..

The result is that seminal plasma is rich in its content of glutamic acid, and are able to design inhibitors to enhance the activity of the non degraded normal substrate if its increased level will have a biologic desired activity. Also biologic activity can be measured to see how it correlates wit the level of message. Tissue may be examined for activity directly rather than indirectly using in-situ analysis or immunohistochemical probes. Because there is another gene highly similar on the other arm of chromosome 11 when isolated the expressed cloned genes can be used to determine what are the substrate differences and use those substrates for identification of PSM related activity, say in circulating cells when looking for metastases.

EXAMPLE 13:

IONOTROPICGLUTAMATE RECEPTOR DISTRIBUTION IN PROSTATE TISSUE

Introduction:

Excitatory neurotransmission in the central nervous system (CNS) is mediated predominantly by glutamate receptors. Two types of glutamate receptors have been identified in human CNS: metabotropic receptors, which are coupled to second-messenger systems, and ionotropic receptors, which serve as ligand-gated ion channels. The presence of ionotropic glutamate receptors in human prostate tissue was investigated.

Methods:

Detection of glutamate receptor expression was performed using anti-GluR2/3 and anti-biotin

immunohistochemical technique in paraffin-embedded human prostate tissues. PSM antigen is a neurocarboxypeptidase that acts to release glutamate. In the CNS glutamate acts as a neurotransmitter by acting on glutaminergic ion channels and increases the flow of ions like calcium ions. One way the glutamate signal is transduced into cell activity is the activation of nitric oxide synthase, and nitric oxide synthase has recently been found to be present in human prostatic tissue. NO is a major signalling mechanism and is involved in control of cell growth and death, in response to inflammation, in smooth muscle cell contraction, etc,. In the prostate much of the stroma is smooth muscle. It was discovered that the prostate is rich in glutaminergic receptors and have begun to define this relationship. Stromal abnormalities are the key feature of BPH. Stromal epithelial interactions are of importance in bothe BPH and CaP. The other glutaminergic receptors through G proteins to change the metabolism of the cell.

Results:

Anti-GluR2/3 immunoreactivity was unique to prostatic stroma and was absent in the prostatic epithelial compartment. Strong anti-GluR4 immunoreactivity was observed in basal cells of prostatic acini.

Discussion:

The differential distribution of ionotropic glutamate receptor subtypes between the stromal and epithelial compartments of the prostate has not been previously described. Prostate-specific membrane antigen (PSMA)has an analogous prostatic distribution, with expression restricted to the epithelial compartment.

PSM antigen is a neurocarboxypeptidase that acts to

release glutamate from NAAG 1, also a potential nerotransmitter. In the CNS glutamate acts as a neurotransmitter by acting on glutaminergic ion channels and increases the flow of ions like calcium ions. One way the glutamate signal is transduced into cell activity is the activation of nitric oxide synthase, and nitric oxide synthase has recently been found to be present in human prostatic tissue. NO is a major signaling mechanism and is involved in control of cell growth and death, in response to inflammation, in smooth muscle cell contraction, etc,. In the prostate much of the stroma is smooth muscle. The prostate is rich in glutaminergic receptors. Stromal abnormalities are the key feature of BPH. Stromal epithelial interactions are of importance in both BPH and CaP. The other glutaminergic receptors through G proteins to change the metabolism of the cell. Glutamate can be produced in the cerebral cortex through the carboxypeptidase activity of the prostate-specific membrane antigen (PSMA). In this location, PSMA cleaves glutamate from acetyl-aspartyl-glutamate. Taken together, these observations suggest a function for PSMA in the human prostate; glutamate may be an autocrine and/or paracrine signalling molecule, possibly mediating epithelial-stromal interactions. Ionotropic glutamate receptors display a unique compartmental distribution in the human prostate.

The carboxypeptidase like activity and one substrate is the dipeptide N-acetyl-aspartyl glutamic acid, NAAG which is one of the best substrates found to date to act as a neurotransmitter in the central nervous system and its abnormal function may be associated with neurotoxic disorder such as epilepsy, ALS, alzheimers etc. PSM carboxypeptidase may serve to process neuropeptide transmitters in the prostate. Neuropeptide transmitters are associated with the

neuroendocrine cells of the prostate and neuroendocrine cells and are thought to play a role in prostatic tumor progression. Interestingly PSM antigen's expression is upregulated in cancer. Peptides known to act as prostatic growth factors such as TGF-a and bFGF, up regulate the expression of the antigen. TNF on the other hand downregulate PSM. TGF and FGF act through the mitogen activated signaling pathway, while TNF acts through the stress activated protein kinase pathway. Thus modulation of PSM expression is useful for enhancing therapy.

EXAMPLE 14:

IDENTIFICATION OF A MEMBRANE-BOUND PTEROYLPOLYGAMMA-GLUTAMYL CARBOXYPEPTIDASE (FOLATE HYDROLASE) THAT IS EXPRESSED IN HUMAN PROSTATIC CARCINOMA

PSM may have activities both as a folate hydrolase and a carboxyneuropeptidase. For the cytotoxic drug methotrexate to be a tumor toxin it has to get into the cell and be polygammaglutamated which to be active, because polyglutamated forms serve as the enzyme substrates and because polyglutamated forms or toxins are also retained by the cell. Folate hydrolase is a competing reaction and deglutamates methotrexate which then can diffuse back out of the cell. Cells that overexpose folate hydrolase activity are resistant to methotrexate. Prostate cancer has always been absolutely refractory to methotrexate therapy and this may explain why, since the prostate and prostate cancer has a lot of folate hydolase activity. However, based on this activity, prodrugs may be generated which would be activate at the site of the tumor such as N-phosphonoacetyl-l-aspartate-glutamate. PALglu is an inhibitor of the enzyme activity with NAAG as a substrate.

Prostate specific membrane antigen was immuno precipitated from the prostate cancer cell line LNCaP and demonstrated it to be rich in folate hydolase activity, with gammaglutamated folate or polyglutamated methotrexate being much more potent inhibitors of the neuropeptidase activity than was quisqualate, which was the most potent inhibitor reported up to this time and consistent with the notion that polyglutamated folates may be the preferred substrate.

Penta-gammaglutamyl-folate is a very potent inhibitor of activity (inhibition of the activity of the enzyme is with 0.5um Ki.) As penta-gammaglutamyl-folate may also be a substrate and as folates have to be depolygammaglutamated in order to be transported into the cell, this suggest that this enzyme may also play a role in folate metabolism. Folate is necessary for the support of cell function and growth and thus this enzyme may serve to modulate folate access to the prostate and prostate tumor. The other area where PSM is expressed is in the small intestine. It turns out that a key enzyme of the small intestine that is involved in folate uptake acts as a gamma-carboxypeptidase in sequentially proteolytically removing the terminal gammaglutaminyl group from folate. In the bone there is a high level of unusual gammaglutamate modified proteins in which the gamma glutamyl group is further carboxylated to produce gammacarboxyglutamate, or GLA. One such protein is osteonectin.

Using capillary electrophoresisis pteroyl poly-gamma-glutamate carboxypeptidase (hydrolase) activity was investigated in membrane preparations from androgen-sensitive human prostatic carcinoma cells (LNCaP). The enzyme immunologically cross-reacts with a derivative of an anti-prostate monoclonal antibody (7E11-C5) that

recognizes prostate specific membrane (PSM) antigen. The PSM enzyme hydrolyzes gamma-glutamyl linkages and is an exopeptidase as it liberates progressively glutamates from methotrexate triuglutamate ($MTXGlu_3$) and folate pentaglutamate (Pte Glu_3) with accumulation of MTX and Pte Glu respectively. The semi-purified membrane-bound enzyme has a broad activity from pH 2 to 10 and is maximally active at pH4.0. Enzymatic activity was weakly inhibited by dithfothreitol ($\geq$0.2 mM) but not by reduced glutathione, homocysteine, or p-hydroxymercuribenzoate (0.05-0.5 mM). By contrast to LNCaP cell membranes, membranes isolated from androgen-insensitive human prostate (TSU-Prl, Duke-145, PC-3) and estrogen-sensitive mammary adenocarcinoma (MCF-7) cells do not exhibit comparable hydrolase activity nor do they react with 7E11-C5. Thus, a folate hydrolase was identified in LNCap cells that exhibits exopeptidase activity and is strongly expressed by these cells.

PALA-Glutamate 3 was tested for efficacy of the prodrug strategy by preparing N-acetylaspartylglutamate, NAAG 1(Figure 59). NAAG was synthesized from commercially available gamma-benzylaspartate which was acetylated with acetic anhydride in pyridine to afford N-acetyl-gamma-benzyl aspartate in nearly quantitative yield. The latter was activated as its pentafluorophenyl ester by treatment with pentafluorophenyltrifluoroacetate in pyridine at 0 deg.C for an hour. This activated ester constitutes the central piece in the preparation of compounds 1 and 4 (Figure 60). When 6 is reacted with epsilon-benzyl-L-glutamate in the presence of HOAT(1-hydroxy-7-azabenzotriazole) in THF-DMF (tetrahydrofuran, N,N- dimethylformamide) at reflux for an overnight period and after removal of the benzyl protecting groups by hydrogenolysis (H2, 30 psi, 10% Pd/C in ethylacetate) gave a product which was

identical in all respects to commercially available NAAG (Sigma).

PALA-Glutamate 3 and analog 5, was synthesized in a similar manner with the addition to the introduction of a protected phosphonoacetate moiety instead of a simple acetate. It is compatible with the function of diethylphosphonoacetic acid which allows the removal of the ethyl groups under relatively mild conditions.

Commercially available diethylphosphonoacetic acid was treated with perfluorophenyl acetate in pyridine at 0 deg.C to room temperature for an hour to afford the corresponding pentafluorophenyl ester in nearly quantitative yield after short path column chromatography. This was then reacted with gamma-benzylaspartate and HOAT in tetrahydrofuran for half an hour at reflux temperature to give protected PALA 7 (N-phosphonoacetylaspartate) in 90% yield after flash column chromatography. The free acid was then activated as its pentafluorophenyl ester 8, then it was reacted with delta-benzyl-L-glutamate and HOAT in a mixture of THF-DMF (9:1, v/v) for 12 hours at reflux to give fully protected PALA-Glutamate 9 in 66% yield after column chromatography. Sequential removal of the ethyl groups followed by the debenzylation was accomplished for a one step deprotection of both the benzyl and ethyl groups. Hence protected PALA-Glutamate was heated up to reflux in neat trimethylsilylchloride for an overnight period. The resulting bistrimethylsilylphosphonate ester 10 was submitted without purification to hydrogenolysis (H_2, 30 psi, 10% Pd/C, ethylacetate). The desired material 3 was isolated after purification by reverse phase column chromatography and ion exchange resin.

Analogs 4 and 5 were synthesized by preparation of

phosphonoglutamate 14 from the alpha-carboxyl-protected glutamate.

Commercially available alpha-benzyl-N-Boc-L-glutamate 11 was treated at refluxing THF with neat boranedimethylsulfide complex to afford the corresponding alcohol in 90% yield. This was transformed into bromide 12 by the usual procedure (Pph_3,CBr_4).

The Michaelis-Arbuzov reaction using triethylphosphite to give the corresponding diethylphosphonate 13 which would be deprotected at the nitrogen with trifluoroacetic acid to give free amine 14. The latter would be condensed separately with either pentafluorophenylesters 6 or 8 to give 16 and 15 respectively, under conditions similar to those described for 3. 15 and 16 would be deprotected in the same manner as for 3 to yield desired analogs 4 and 5.

An inhibitor of the metabolism of purines and pyrimidine like DON (6-diazo-5-oxo-norleucine) or its aspartate-like 17, and glutamate-like 18 analogs would be added to the series of substrates.

Analog 20 is transformed into compound 17 by treatment with oxalyl chloride followed by diazomethane and deprotection under known conditions to afford the desired analogs. In addition, azotomycin is active only after in vivo conversion to DON which will be released after action of PSM on analogs 17, 18, and 19.

In addition, most if not all chemotherapies rely on one hypothesis; fast growing cells possess a far higher appetite for nutrients than normal cells. Hence, they uptake most of the chemotherapeutic drugs in their proximity. This is why chemotherapy is associated with

serious secondary effects (weakening of the immune system, loss of hair, ...) that sometimes put the patient's life in danger. A selective and effective drug that cures where it should without damaging what it shouldn't damage is embodied in representative structures 21 and 22.

Representative compounds, 21 and 22, were designed based on some of the specific effects and properties of PSM, and the unique features of some newly discovered cytotoxic molecules with now known mode of action. The latter, referred to commonly as enediynes, like dynemycin A 23 and or its active analogs. The recent isolation of new natural products like Dynemycin A 23, has generated a tremendous and rapidly growing interest in the medical and chemical sciences. They have displayed cytotoxicities to many cancer cell lines at the sub-nanomolar level. One problem is they are very toxic, unstable, and non-selective. Although they have been demonstrated, in vitro, to exert their activity through DNA damage by a radical mechanism as described below, their high level of toxicity might imply that they should be able to equally damage anything in their path, from proteins to enzymes, ...etc.

These molecules possess unusual structural features that provide them with exceptional reactivities. Dynemycin A 23 is relatively stable until the anthraquinone moiety is bioreduced into hydroanthraquinone 24. This triggers a chain of events by which a diradical species 25 is generated as a result of a Bergman cycloaromatization[F]. Diradical species 25 is the ultimate damaging edge of dynemycin A. It subtracts 2(two) protons from any neighboring molecule or molecules(ie. DNA) producing radicals therein. These radicals in turn combine with molecular oxygen to give hydroperoxide intermediates that, in the

case of DNA, lead to single and double strand incision, and consequent cell death. Another interesting feature was provided by the extensive work of many organic chemists who not only achieved the total synthesis of (+)-dynemycin A 23 and other enediynes. but also designed and efficiently prepared simpler yet as active analogs like 26.

Enediyne 26 is also triggerable and acts by virtue of the same mechanism as for 23. This aspect is very relevant to the present proposed study in that 27 (a very close analog of 26) is connected to NAAG such that the NAAG-27 molecule, 21, would be inert anywhere in the body (blood, organs, normal prostate cells, ...etc.) except in the vicinity of prostate cancer, and metastatic cells. In this connection NAAG plays a multiple role:

- Solubilization and transport: analogs of 26-type are hydrophobic and insoluble in aqueous media, but with a water soluble dipeptide that is indigenous to the body, substrate 21 should follow the ways by which NAAG is transported and stored in the body.

- Recognition, guidance, and selectivity: Homologs of PSM are located in the small intestines and in the brain.

In the latter, a compound like 27 when attached to a multiply charged dipeptide like NAAG, has no chance of crossing the blood brain barrier. In the former case, PSM homolog concentration in the small intestines is very low compared to that of PSM in prostrate cancer cells. In addition, one could enhance the selectivity of delivery of the prodrug by local injection in the prostate. Another image of this strategy could be formulated as follows. If prostate cancer were a war

in which one needed a "smart bomb" to minimize the damage within the peaceful surroundings of the war zone, then 21 would be that "smart bomb". NAAG would be its guidance system, PSM would be the trigger, and 27 would be the warhead.

26 and its analogs are established active molecules that portray the activity of dynemycin A. Their syntheses are described in the literature. The total synthesis of optically active 27 has been described[G]. The synthetic scheme that for the preparation of 28 is almost the same as that of 27. However, they differ only at the position of the methoxy group which is meta to the nitrogen in the case of 28. This requires an intermediate of type 29, and this is going to be prepared by modification of the Myers' method. Compound 28 is perhaps the closest optically active analog that resembles very much 26, and since the activity of the latter is known and very high.

Since NAAG is optically pure, its combination with racemic material sometimes complicates purification of intermediates. In addition, to be able to modify the components of this system one at a time, optically pure intermediates of the type 21 and 22 are prepared. 27 was prepared in 17 steps starting fro commercially available material. Another interesting feature of 27 is as demonstrates in a very close analog 26, it possesses two(2) triggers as shown by the arrows.

The oxygen and the nitrogen can both engender the Bergman cycloaromatization and hence the desired damage. The simple protection deprotection manipulation of either functionality should permit the selective positioning of NAAG at the nitrogen or at the oxygen centers. PSM should recognize the NAAG portion of 21 or 22, then it would remove the glutamic acid

moiety. This leaves 27 attached to N-acetylaspartate.

Intramolecular assisted hydrolysis of systems like N-acetylaspartyle is well documented in the literature. The aminoacid portion should facilitate the hydrolysis of such a linkage. In the event this would not work when NAAG is placed on the nitrogen, an alternative would be to attach NAAG to the oxygen giving rise to phenolic ester 22 which is per se labile and removable under milder conditions. PSM specific substrates can be designed that could activate pro-drugs at the site of prostatic tumor cells to kill those cells. PSM specific substrates may be used in treatment of benign prostatic hyperplasia.

EXAMPLE 15:

GENOMIC ORGANIZATION OF PSM EXON/INTRON JUNCTION SEQUENCES

EXON 1 Intron 1

1F. strand

CGGCTTCCTCTTCGG

cggcttcctcttcgg taggggggcgcctcgcggag...tatttttca

1R. strand ...ataaaaagtCCCACCAAA

Exon 2 Intron 2

2F. strand

ACATCAAGAAGTTCT

acatcaagaagttct caagtaagtccatactcgaag...

2R. strand ...caagtggtcATTAAAATG

Exon 3 Intron 3

3F. strand

GAAGATGGAAATGAG

gaagatggaaatgag gtaaaatataaataaataaataa...

Exon 4 Intron 4

4F. strand

AAGGAATGCCAGAGG

aaggaatgccagagg taaaaacacagtgcaacaaa...

4R. strand ...agagttgTCCCGCTAGAT

Exon 5 Intron 5

5F. strand

CAGAGGAAATAAGGT

cagaggaaataaggt aggtaaaaattatctctttttt...

...gtgttttctAGGTTAAAAATG

5R. strand ...cacttttgaTCCAATTT

Exon 6 Intron 6

6F. strand

GTTACCCAGCAAATG

gttacccagcaatg gtgaatgatcaatccttgaat...

6R. strand ...aaaaaaagtCTTATACGAATA

Exon 7 Intron 7

7F. strand

ACAGAAGCTCCTAGA

acagaagctcctaga gtaagtttgtaagaaaccargg...

7R. strand ...aaacacaggttatcTTTTTACCCA

Exon 8 Intron 8

8F. strand

AAACTTTTCTACACA

aaacttttctacaca gttaagagactatataaatttta...

8R. strand ....aaacgtaatcaTTTTCAGTTCTAC

Exon 9 Intron 9

9F. strand

AGCAGTGGAACCAG

agcagtggaaccag gtaaaggaatcgtttgctagca...

...tttctagatAGATATGTCATTC

9R. strand ...aaagaTCTGTCTATACAGTAA

Exon 10 Intron 10

10F. Strand

CTGAAAAAGGAAGG

ctgaaaaaggaagg taatacaaacaaatagcaagaa...

Exon 11 Intron 11

11F. Strand

TGAGTGGGCAGAGG

agagg ttagttggtaatttgctataatata...

Exon 13 Intron 12

12R. strand

GAGTGTAGTTTCCT

gtagtttcct gaaaaataagaaaagaatagat...

Exon 14 Intron 13

13R. strand

AGGGCTTTTCAGCT

agggcttttcagct acacaaattaaaagaaaaaaag...

Exon 14 Intron 14

14F. strand

GTGGCATGCCCAGG

gtggcatgcccagg taaataaatgaatgaagtttcca...

Exon 16 Intron 15

15R. strand

AATTTGTTTGTTTCC

aatttgtttgtttcc tacagaaaaaacaacaaaaca...

Exon 16 Intron 16

16F. strand

CAGTGTATCATTTG

cagtgtatcatttg gtatgttacccttccttttcaaatt...

...tttcagATTCACTTTTTT

16R. strand ...aaagtcTAAGTGAAAA

Exon 17 Intron 17

17F. strand

TTTGACAAAAGCAA

tttgacaaaagcaa gtatgttctacatatatgtgcatat...

17R. strand ...aaagagtcGGGTTA

Exon 18 Intron 18

18F. strand

GGCCTTTTTATAGG

ggcctttttatagg taaganaagaaaatatgactcct...

18R. strand ...aatagttgTGTAAACCC

Exon 19 Intron 19

19F. strand

GAATATTATATATA

gaatattatatata gttatgtgagtgtttatatatgtgtgt...

Notes: F: Forward strand

R: Reverse strand

What is claimed is:

1. An isolated nucleic acid molecule encoding an alternatively spliced prostate-specific membrane (PSM') antigen.

2. An isolated mammalian DNA molecule of claim 1.

3. An isolated mammalian cDNA molecule of claim 2.

4. An isolated mammalian RNA molecule derived from claim 1.

5. An isolated nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the isolated nucleic acid molecule of claim 1.

6. A DNA molecule of claim 5.

7. A RNA molecule of claim 5.

8. A method of detecting expression of a alternatively spliced prostate-specific membrane (PSM') antigen in a cell which comprises obtaining total mRNA from the cell, contacting the mRNA so obtained with a labelled nucleic acid molecule of claim 5 under hybridizing conditions, determining the presence of mRNA hybridized to the molecule, and thereby detecting the expression of the alternatively spliced prostate-specific membrane (PSM') antigen in the cell.

9. An isolated nucleic acid molecule of claim 2 operatively linked to a promoter of RNA transcription.

10. A vector which comprises the isolated nucleic acid molecule of claim 1.

11. A host vector system for the production of a polypeptide having the biological activity of the alternatively spliced prostate-specific membrane (PSM') antigen which comprises the vector of claim 10 and a suitable host.

12. A host vector system of claim 11, wherein the suitable host is a bacterial cell, insect cell, or mammalian cell.

13. A method of producing a polypeptide having the biological activity of the prostate-specific membrane antigen which comprises growing the host cells of the host vector system of claim 12 under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced.

14. An isolated nucleic acid molecule encoding a prostate-specific membrane antigen promoter.

15. A polypeptide encoded by the isolated nucleic acid molecule of claim 1.

16. A method of detecting hematogenous micrometastic tumor cells of a subject, comprising (A) performing nested polymerase chain reaction (PCR) on blood, bone marrow or lymph node samples of the subject using the prostate specific membrane antigen primers, and (B) verifying micrometastases by DNA sequencing and Southern analysis, thereby detecting hematogenous micrometastic tumor cells of the subject.

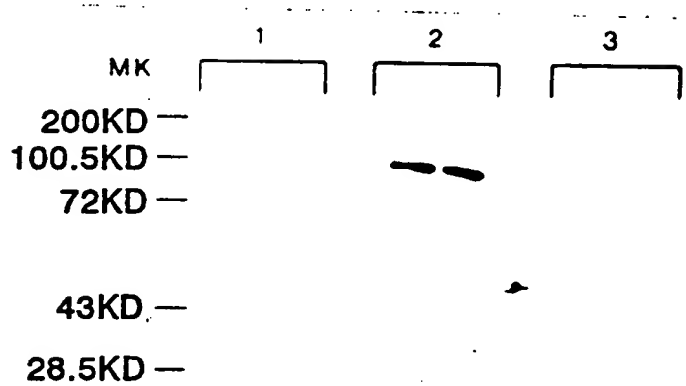
17. The method of claim 16, wherein the primers are derived from prostate specific antigen.

18. The method of claim 16, wherein the subjects is administered hormones, epidermal growth factor, b-fibroblast growth factors, or tumor necrosis factor.

19. A method of determining prostate cancer progression in a subject which comprises: a) obtaining a suitable prostate tissue sample; b) extracting RNA from the prostate tissue sample; c) performing a RNAse protection assay on the RNA, thereby forming a duplex RNA-RNA hybrid; d) detecting PSM and PSM' amounts in the tissue sample; e) calculating a PSM/PSM' tumor index, thereby determining prostate cancer progression in the subject.

20. The method of claim 19, further comprising performing in-situ hyribridization.

FIGURE 1



1 - anti- EGFr PoAB RK-2
2 - Cyt-356 MoAB/RAM
3 - RAM

FIGURE 2A

FIGURE 2B

FIGURE 2C

FIGURE 2D

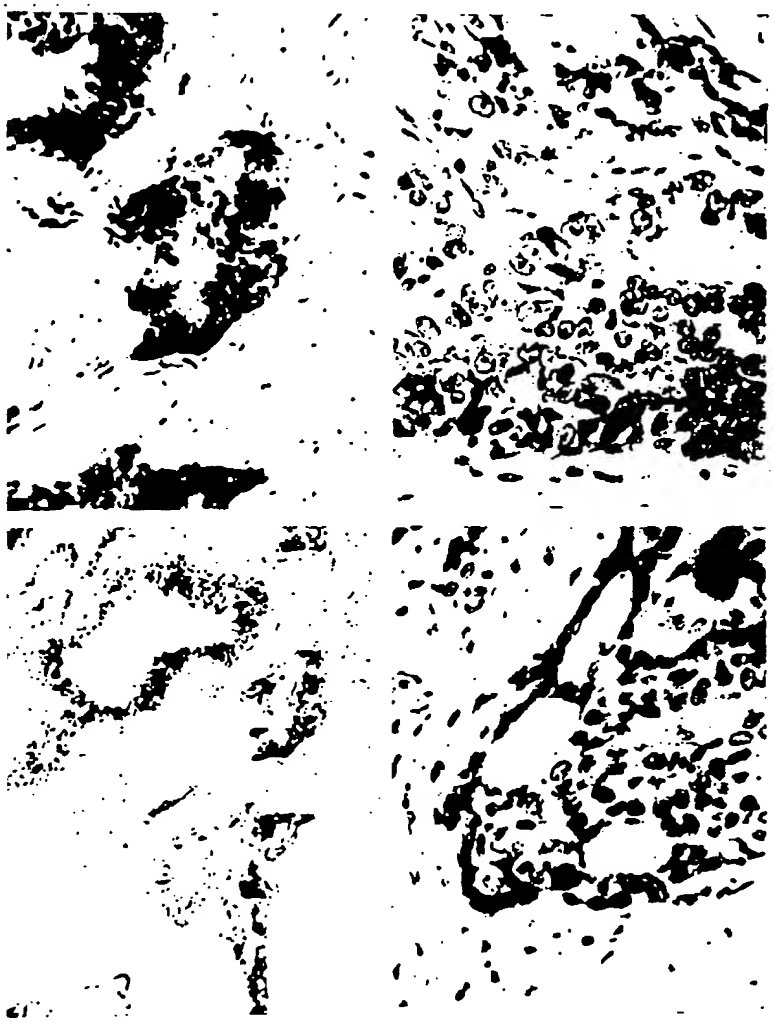


FIGURE 3A

FIGURE 3B

FIGURE 3C

FIGURE 3D

FIGURE 4

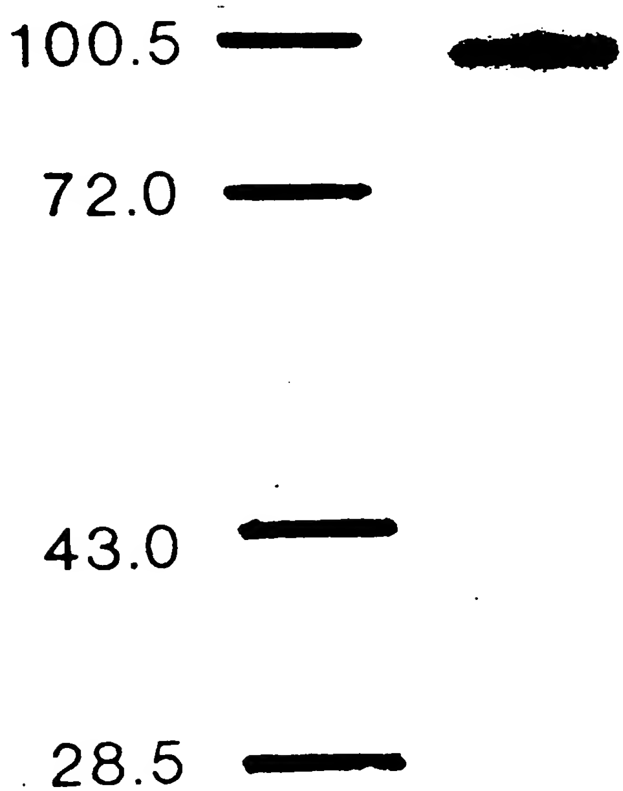


FIGURE 5

FIGURE 6A FIGURE 6B

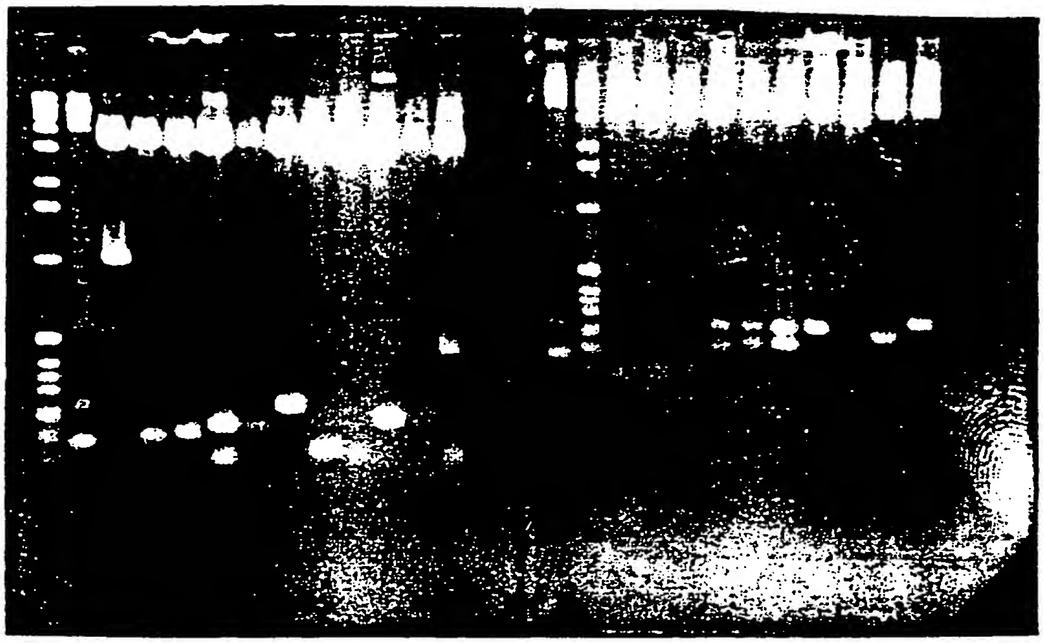


FIGURE 7

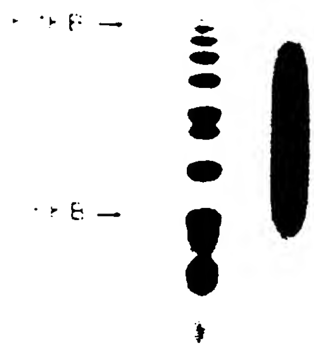


FIGURE 8

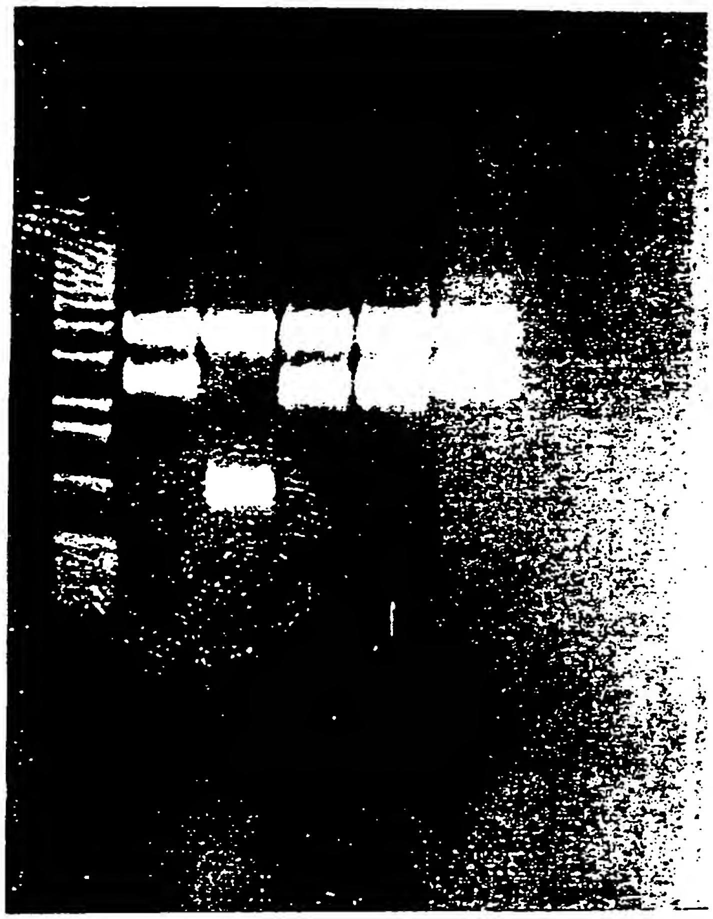


FIGURE 9

4 —
3 —
2 —
1.6 —

FIGURE 10

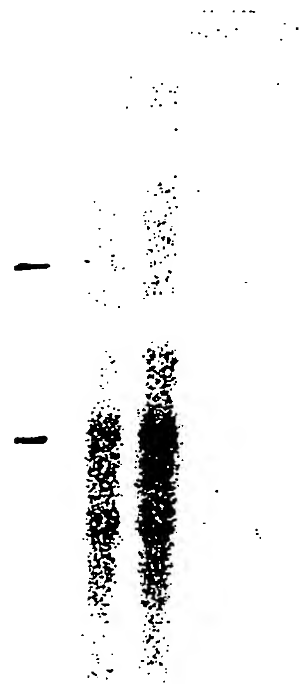


FIGURE 11

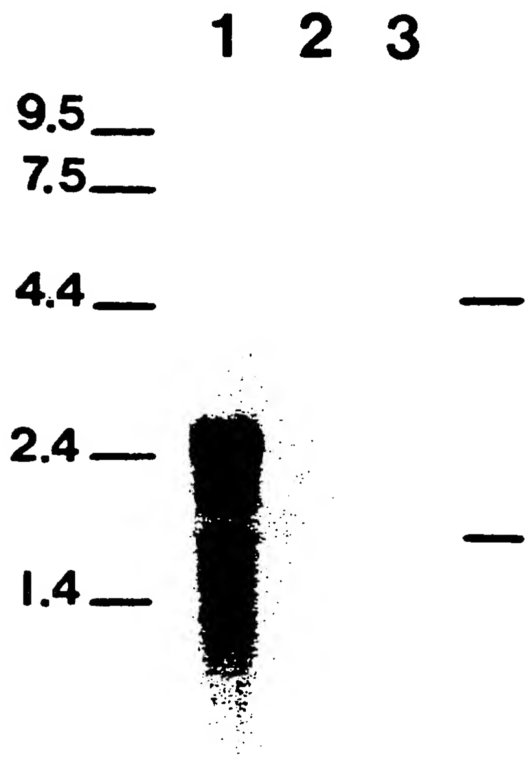


FIGURE 12A FIGURE 12B

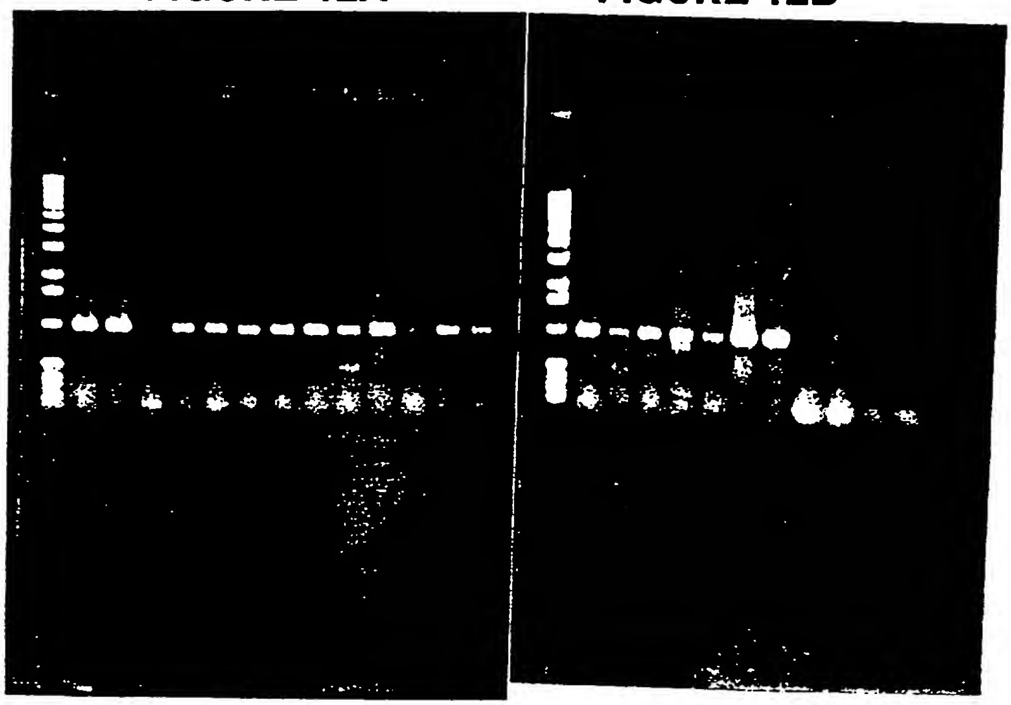


FIGURE 13

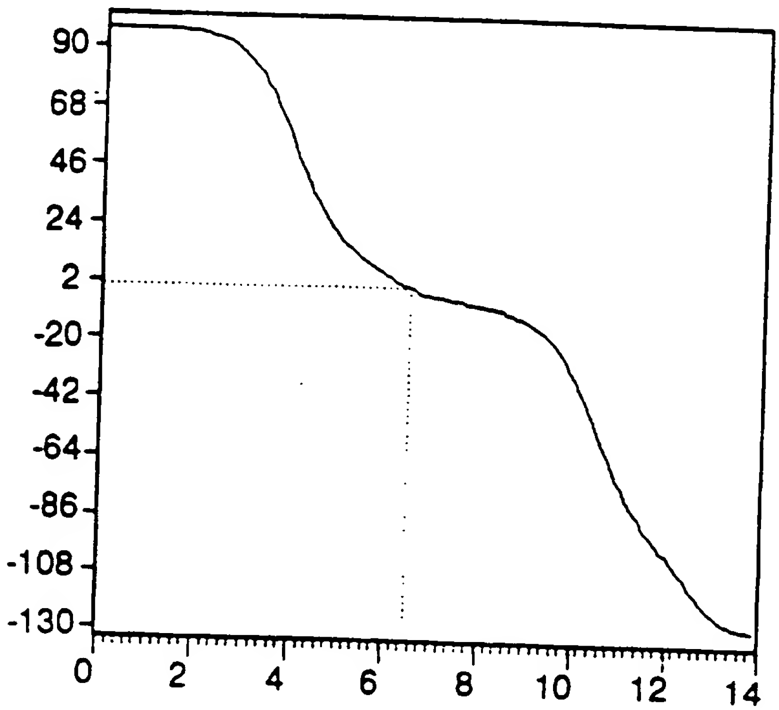


FIGURE 14-1

```
Done on sequence PMSANTIGEN.
Total number of residues is: 750.
Analysis done on the complete sequence.

In Helical   (H) conformation [DC =  -75 CNAT ] :  264 AA => 35.2%
In Extended  (E) conformation [DC =  -88 CNAT ] :  309 AA => 41.2%
In   Turn    (T) conformation [DC =    0 CNAT ] :   76 AA => 10.1%
In   Coil    (C) conformation [DC =    0 CNAT ] :  101 AA => 13.4%

               ---------------------------------------

Sequence shown with conformation codes.
=======================================

Consecutive stretch of 5 or more residues in a given conformation are
overlined.

  1  H H H H H H H H H H H H H H E E E T T E E E E E E E E E E E
 31  E E E E E E H H H H H H C C C C C C T H H H H H H H H H H H
 61  H H H H H H H H H H H H E E E T T E E E E C C C C C H H H H
```

FIGURE 14-2

91 $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ E H C E E E T T C C $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ T

121 T T C C $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ C T C C H $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ T T C C C C T T

151 T $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ T E C C T C C C $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{H}$

181 $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ T T T $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$

211 T T C C $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ T T $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ T E E

241 E E T T T T E C C T T C $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ T T T C $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$

271 C C C $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ H H $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ C C $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$

301 $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ E T T T C C C C T E T T E T $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$

331 $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ C E C $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ E E E C C C C $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$

361 $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ C C C T E E E T T C C C T C

391 C H H H E E E E $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ C C C T T T C C C T $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ C

421 $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ C C C C $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$

FIGURE 14-3

451 E E E C C E E T T E E E E E E E E E E E E E H H H H H H H H

481 $\bar{H}$ C C $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ T T T $\bar{C}$ $\bar{C}$ $\bar{C}$ $\bar{C}$ $\bar{C}$ T $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$

511 $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{C}$ $\bar{C}$ $\bar{C}$ $\bar{C}$ $\bar{C}$ E E E $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ T C C C E E E T T E C T

541 T E $\bar{T}$ $\bar{T}$ $\bar{T}$ $\bar{T}$ $\bar{T}$ C $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$

571 $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ E H $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ T $\bar{H}$ $\bar{H}$ $\bar{H}$

601 $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ H C C $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$

631 $\bar{H}$ T T T C C $\bar{E}$ $\bar{E}$ $\bar{E}$

661 $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ E E T T C C C T $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$

691 $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ T C C C C T $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ T E E $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ C C

721 C $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{E}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$ $\bar{H}$

FIGURE 14-4

```
Semi-graphical output.
======================

Symbols used in the semi-graphical representation:

Helical  conformation: X       Extended conformation: -
  Turn   conformation: >         Coil   conformation: *

         10        20        30        40        50
          |         |         |         |         |
MWNLLHETDSAVATARRPRWLCAGALVLAGGFFLLGFLFGWFIKSSNEAT

XXXXXXXXXXXXXX--->>-----------------XXXXXX******>X
XXXXXXXXXXXXXX--->>-----------------XXXXXX******>X

         60        70        80        90       100
          |         |         |         |         |
NITPKHNMKAFLDELKAENIKKFLYNFTQIPHLAGTEQNFQLAKQIQSQW
```

FIGURE 14-5

```
XXXXXXXXXXXXXXXXXXXXXX--->>----*****XXXXXXXXX-X*--
XXXXXXXXXXXXXXXXXXXXXX--->>----*****XXXXXXXXX-X*--

         110       120       130       140       150
           |         |         |         |         |
KEFGLDSVELAHYDVLLSYPNKTHPNYISIINEDGNEIFNTSLFEPPPPG

->>**XXXXXXXXX----->>>**--------*>**X----->>****>>
->>**XXXXXXXXX----->>>**--------*>**X----->>****>>

         160       170       180       190       200
           |         |         |         |         |
YENVSDIVPPFSAFSPQGMPEGDLVYVNYARTEDFFKLERDMKINCSGKI
```

FIGURE 14-6

```
>----------->-**>***---------XXXXXXXXXXXXXXX>>>---
>----------->-**>***---------XXXXXXXXXXXXXXX>>>---

        210       220       230       240       250
         |         |         |         |         |
VIARYGKVFRGNKVKNAQLAGAKGVILYSDPADYFAPGVKSYPDGWNLPG

---------->>**XXXXXXXX-------->>----->---->>>>-**>
---------->>**XXXXXXXX-------->>----->---->>>>-**>

        260       270       280       290       300
         |         |         |         |         |
GGVQRGNILNLNGAGDPLTPGYPANEYAYRRGIAEAVGLPSIPVHPIGYY

>*--------->>>*-----***-----XX--------**----------
>*--------->>>*-----***-----XX--------**----------

        310       320       330       340       350
         |         |         |         |         |
DAQKLLEKMGGSAPPDSSWRGSLKVPYNVGPGFTGNFSTQKVKMHIHSTN

XXXXXXX->>>****>->>->-------------*-*XXXXXX---****
XXXXXXX->>>****>->>->-------------*-*XXXXXX---****

        360       370       380       390       400
         |         |         |         |         |
EVTRIYNVIGTLRGAVEPDRYVILGGHRDSWVFGGIDPQSGAAVVIIEIVR
```

FIGURE 14-7

```
-------------------------***>---->>***>**XXX----XX
-------------------------***>---->>***>**XXX----XX

        410       420       430       440       450
         |         |         |         |         |
SFGTLKKEGWRPRRTILFASWDAEEFGLLGSTEWAEENSRLLQERGVAYI

XXX***>>>***>-------*XXXXXXXX****XXXXXXXXXXX------
XXX***>>>***>-------*XXXXXXXX****XXXXXXXXXXX------

        460       470       480       490       500
         |         |         |         |         |
NADSSIEGNYTLRVDCTPLMYSLVHNLTKELKSPDEGFEGKSLYESWTKK

---**-->>-------------XXXXXXXXX**XXXXXXXXXXXXX>>>*
---**-->>-------------XXXXXXXXX**XXXXXXXXXXXXX>>>*

        510       520       530       540       550
         |         |         |         |         |
SPSPEFSGMPRISKLGSGNDFEVFFQRLGIASGRARYTKNWETNKFSGYP

****>----------*****---XXXXX>***--->>-*>>->>>>>*--
****>----------*****---XXXXX>***--->>-*>>->>>>>*--

        560       570       580       590       600
         |         |         |         |         |
```

FIGURE 14-8

```
LYHSVYETYELVEKFYDPMFKYHLTVAQVRGGMVFELANSIVLPFDCRDY

------XXXXXXXXXXXXXXXXXX-X---------XXXXX------>XXX
------XXXXXXXXXXXXXXXXXX-X---------XXXXX------>XXX

         610       620       630       640       650
           |         |         |         |         |
AVVLRKYADKIYSISMKHPQEMKTYSVSFDSLFSAVKNFTEIASKFSERL

XXXXXXXXXX-----X**XXXXX-------XXXXXXXXXXXXXXXXXXXX
XXXXXXXXXX-----X**XXXXX-------XXXXXXXXXXXXXXXXXXXX

         660       670       680       690       700
           |         |         |         |         |
QDFDKSNPIVLRMMNDQLMCLERAFIDPLGLPDRPFYRHVIYAPSSHNKY

XX>>>**------XXXXXXXXXXXX-->>***>----------->****>
XX>>>**------XXXXXXXXXXXX-->>***>----------->****>

         710       720       730       740       750
           |         |         |         |         |
AGESFPGIYDALFDIESKVDPSKAWGEVKRQIYVAAFTVQAAAETLSEVA

------>--XXXXXXXXX***XXXXXXXX--------XXXXXXXXXXXXX
------>--XXXXXXXXX***XXXXXXXX--------XXXXXXXXXXXXX
```

FIGURE 15A

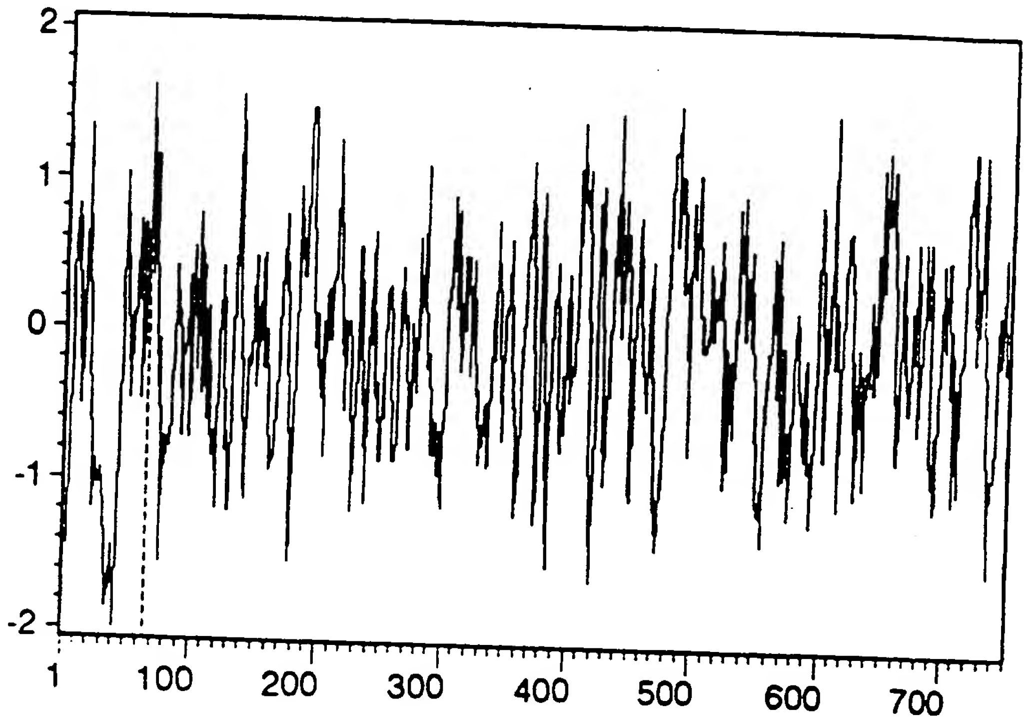


FIGURE 15B

```
*****************************************
* PREDICTION OF ANTIGENIC DETERMINANTS *
*****************************************

Done on sequence PMSANTIGEN.
Total number of residues is: 750.
Analysis done on the complete sequence.

The method used is that of Hopp and Woods.
The averaging group length is: 6 amino acids.
-> This is the value recommended by the authors <-

                 ----------------------------------------

The three highest points of hydrophilicity are:

( 1)   Ah= 1.62 : From   63 to   68 : Asp-Glu-Leu-Lys-Ala-Glu
( 2)   Ah= 1.57 : From  132 to  137 : Asn-Glu-Asp-Gly-Asn-Glu
( 3)   Ah= 1.55 : From  482 to  487 : Lys-Ser-Pro-Asp-Glu-Gly

Ah stands for: Average hydrophilicity.

Note that, on a group of control proteins, only the highest point was in 100%
of the cases assigned to a known antigenic group. The second and third points
gave a proportion of 33% of incorrect predictions.
```

FIGURE 16-1

```
The best scores are:                                              initn init1   opt
CHKTFER   G.gallus mRNA for transferrin receptor                    203   120   321
RATTRFR   Rat transferrin receptor mRNA, 3' end.                    164   164   311
HUMTFRR   Human transferrin receptor mRNA, complete cd              145   145   266


CHKTFER   G.gallus mRNA for transferrin receptor                    203   120   321
  51.9% identity in 717 nt overlap

           1020      1030      1040      1050      1060      1070
pmsgen TGTCCAGCGTGGAAATATCCTAAATCTGAATGGTGCAGGAGACCCTCTCACACCAGGTTA
                                     :::  : :::::::::  ::: ::::: :
CHKTFE TACACTTATCCCATTCGGACATGCCCACCTTGGAACTGGAGACCCTTACACCCCAGGCTT
           990      1000      1010      1020      1030      1040

           1080      1090      1100      1110      1120      1130
pmsgen CCCAGCAAATGAATATGCTTATAGGCGTGGAATTGCAGAGGCTGTTGGTCTTCCAAGTAT
       :::  :     :  :  :  :   :  :  :   :  ::  ::   :: :: ::    ::
CHKTFE CCCTTCGTTCAACCACACCCA---GTTTCCACCAGTTGAATCTTCAGGACTACCCCACAT
           1050      1060           1070      1080      1090      1100

           1140      1150      1160      1170      1180      1190
pmsgen TCCTGTTCATCCAATTGGATACTATGATGCACAGAAGCTCCTAGAAAAATGGGTGGCTC
       : :::::::  : ::      :  ::  :::   : :::  :    ::::::: :::
CHKTFE TGCTGTTCAGACCATCTCTAGCAGTGCAGCAGCCAGGCTGTTCAGCAAAATGGATGGAGA
               1110      1120      1130      1140      1150      1160
```

FIGURE 16-2

```
        1200      1210      1220      1230      1240      1250
pmsgen  AGCACCACCAGATAGCAGCTGGAGAGGAAGTCTCAAAGTGCCCTACAATGTTGGACCTGG
          ::    : :: ::  : :::: :::     :: :     :::  :: ::  :::    
CHKTFE  CACATGCTCTGA-AG--GTTGGAAAGGTGCGATCCA---TTCCTGTAAGGT--GAC--AA
                  1170         1180      1190         1200        1210

        1260      1270      1280      1290      1300      1310
pmsgen  CTTTACTGGAAACTTTTCTACACAAAAAGTCAAGATGCACATCCACTCTACCAATGAAGT
        :    : ::: :         :  ::  :: ::  :  : :  ::  : :::   ::: 
CHKTFE  CAAAGCAGGAGA----GCCAGA-TAATGGTGAAACTAGATGTGAACAATTCCATGAAAGA
                1220           1230      1240      1250      1260

        1320      1330      1340      1350      1360      1370
pmsgen  GACAAGAATTTACAATGTGATAGGTACTCTCAGAGGAGCAGTGGAACCAGACAGATATGT
         :  :  :::   ::  :  : ::: :  ::   :     :  ::::: ::  : :::::
CHKTFE  CAGGAAGATTCTGAACATCTTCGGTGCTATCCAGGGATTTGAAGAACCTGATCGGTATGT
        1270      1280      1290      1300      1310      1320

        1380      1390      1400      1410      1420      1430
pmsgen  CATTCTGGGAGGTCACCGGGACTCATGGGTGTTTGGTGGTATTGACCCTCAGAGTGGAGC
          :  ::::::  ::  : ::::  ::::     ::      : :  ::   : :::: :
CHKTFE  TGTGATTGGAGCCCAGAGAGACTCCTGGGGCCCAGGAGTGGCTAAAGCTGGCACTGGAAC
        1330      1340      1350      1360      1370      1380
```

FIGURE 16-3

```
        1440      1450      1460      1470      1480      1490
pmsgen  AGCTGTTGTTCATGAAATTGTGAG---GAGCTTTGGAACACTGAAAAAGGAAGGGTGGAG
         ::: :  :    ::: :::   :   :: ::  :  : : ::::::: :: :: :  : 
CHKTFE  TGCTATATTGTTGGAACTTGCCCGTGTGATCTCAGACATAGTGAAAAACGAGGGCTACAA
          1390      1400      1410      1420      1430      1440

          1500      1510      1520      1530      1540      1550
pmsgen  ACCTAGAAGAACAATTTTGTTTGCAAGCTGGGATGCAGAAGAATTTGGTCTTCTTGGTTC
        ::: ::  :::  ::  : X:::: ::::::  ::::: ::: :  ::   : : ::: :
CHKTFE  ACCGAGGCGAAGCATCATCTTTGCTAGCTGGAGTGCAGGAGACTACGGAGCTGTGGGTGC
          1450      1460      1470      1480      1490      1500

          1560      1570      1580      1590      1600      1610
pmsgen  TACTGAGTGGGCAGAGGAGAATTCAAGACTCCTTCAAGAGCGTGGCGTGGCTTATATTAA
        :::::: :::   :::: : : :X     : :: :: :     :   :  :::: :: : 
CHKTFE  TACTGAATGGCTGGAGGGGTACTCTGCCATGCTGCATGCCAAAGCTTTCACTTACATCA-
          1510      1520      1530      1540      1550      1560

          1620      1630      1640      1650      1660      1670
pmsgen  TGC-TGACTCATCTATAGAAGGAAACTA-CACTCTGAGAGTTGATTGTACACCGCTGATG
         :: ::  :  :: :     :: : : : :  : : :   ::  :   :  ::  :: ::
CHKTFE  -GCTTGGATGCTCCAGTCCTGGGAGCAAGCCATGTCAAGATTTCTGCCAGCCCCTTGCTG
          1570      1580      1590      1600      1610      1620
```

FIGURE 16-4

```
             1680      1690      1700      1710      1720      1730
pmsgen TACAGCTTGGTACACAACCTAACAAAAGAGCTGAAAAGCCCTGATGAAGGCTTTGAAGGC
       :: :   :: :    :   : :  ::: : :   : :: :: : :  : ::  ::  ::
CHKTFE TATATGCTGCTGGGGAGTATTATGAAGGGGGTGAAGAATCCAGCAGCAGTCTCAGAGAGC
          1630      1640      1650      1660      1670      1680

             1740      1750      1760      1770      1780      1790
pmsgen AAATCTCTTTATGAAAGTTGGACTAAAAAAAGTCCTTCCCCAGAGTTCAGTGGCATGCCC
           :::: ::  : : :::: : : :
CHKTFE ----CTCTATAACAGACTTGGCCCAGACTGGGTAAAAGCAGTTGTTCCTCTTGGCCTGGA
              1690      1700      1710      1720      1730
```

FIGURE 16-5

```
RATTRFR   Rat transferrin receptor mRNA, 3' end.         164   164   311
  55.5% identity in 560 nt overlap

            1210      1220      1230      1240      1250
pmsgen CCACCAGATAGCAGCTGGAGAGGAAGTCTCAAAGTGCCCTACAATGTTGGACCTGGCTT-
                                    ::: :: ::: : :  : : :  : : ::
RATTRF TGCAGAAAAGCTATTCAAAAACATGGAAGGAAACTGTCCTCCTAGTTGGAATATAGATTC
        610       620       630       640       650       660

        1260      1270      1280      1290       1300       1310
pmsgen -TACTGGAAACTTTTCTACACAAAAAGTCAAGATGCACATC-CACTCT-ACCAATG----
        :  :: :: ::          :: : : :::: :::    : : :::: : : :::::
RATTRF CTCATGTAAGCTGGAACTTTCACAGAATCAAAATGTGAAGCTCACTGTGAACAATGTACT
        670       680       690       700       710       720
```

FIGURE 16-6

```
             1320      1330      1340      1350      1360      1370
pmsgen --AAGTGACAAGAATTTACAATGTGATAGGTACTCTCAGAGGAGCAGTGGAACCAGACAG
         :::  ::::::::    ::  :  : ::   : : : :::    : :::::::::: :
RATTRF GAAAGAAACAAGAATACTTAACATCTTTGGCGTTATTAAAGGCTATGAGGAACCAGACCG
     730       740       750       760       770       780

             1380      1390      1400      1410      1420      1430
pmsgen ATATGTCATTCTGGGAGGTCACCGGGACTCATGGGTGTTTGGTGGTATTGACCCTCAGAG
        ::  :  :  : ::::  ::  : ::: : ::::    :::: ::   ::    ::: :
RATTRF CTACATTGTAGTAGGAGCCCAGAGAGACGCTTGGGGCCCTGGT-GTTGCGAAGTCCAGTG
     790       800       810       820       830       840

              1440      1450      1460      1470          1480
pmsgen T-GGAGCAGCTGTTGTTCATGAAATTGTGAGGAGCTTTGGAACA-CTGA---AAAAGGAA
       : ::: ::: : ::  :  ::::: :      ::  ::   : :  :::   ::::: : 
RATTRF TGGGAACAGGTCTT-CTGTTGAAACTTGCCCAAGTATTCTCAGATATGATTTCAAAAGAT
      850       860       870       880       890       900

        1490      1500      1510      1520      1530      1540
pmsgen GGGTGGAGACCTAGAAGAACAATTTTGTTTGCAAGCTGGGATGCAGAAGAATTTGGTCTT
       :: :  X:::: :: :: :  ::: : ::::: ::::::  ::::: ::: : :::   :
RATTRF GGATTTAGACCCAGCAGGAGTATTATCTTTGCCAGCTGGACTGCAGGAGACTATGGAGCT
       910       920       930       940       950       960
```

FIGURE 16-7

```
          1550      1560      1570          1580      1590      1600
pmsgen CTTGGTTCTACTGAGTGGGCAGAGGAGAA---TTCAAGACTCCTTCAAGAGCGTGGCGTG
        ::::: : :::::::::   :::: : X   ::::    : : :: : ::   :   :
RATTRF GTTGGTCCGACTGAGTGGCTGGAGGGGTACCTTTCATCTTTGCATCTAAAG---GCTTTC
       970       980       990      1000      1010          1020

           1610      1620      1630      1640       1650      1660
pmsgen GCTTATATTAATGCTGACTCATCTATAGAAGGAAACTA-CACTCTGAGAGTTGATTGTAC
        :::: :::::: :::  : :  :      ::  :::: ::   : :  :::  :   :
RATTRF ACTTACATTAAT-CTGGATAAAGTCGTCCTGGGTACTAGCAACTTCAAGGTTTCTGCCAG
         1030      1040      1050      1060      1070      1080

           1670      1680      1690      1700      1710        1720
pmsgen ACCGCTGATGTACAGCTTGGTACACAACCTAACAAAAGAGCTGAAAAGC-CCTGATGAAG
        :: ::  : :: :   :  :    ::  :::   : ::   : :::::  : ::
RATTRF CCCCCTATTATATACACTTATGGGAAGATAATGCAGGA--CGTAAAGCATCCGA-----
         1090      1100      1110      1120        1130
```

FIGURE 16-8

```
             1730      1740            1750      1760      1770
pmsgen GCTTTGAAGGCAAATCTCTTTAT-GAA-----AGTTGGACTAAAAAAAGTCCTTCCCCAG
          :::: :: :::: ::: ::: :::     : ::::: ::  :::: :
RATTRF ---TTGATGGAAAATATCTATATCGAAACAGTAATTGGATTAGCAAAATTGAGGAACTTT
                1140      1150      1160      1170      1180      1190

             1780      1790      1800      1810      1820      1830
pmsgen AGTTCAGTGGCATGCCCAGGATAAGCAAATTGGGATCTGGAAATGATTTTGAGGTGTTCT

RATTRF CCTTGGACAATGCTGCATTCCCTTTTCTTGCATATTCAGGAATCCCAGCAGTTTCTTTCT
             1200      1210      1220      1230      1240      1250
```

FIGURE 16-9

```
HUMTFRR   Human transferrin receptor mRNA, complete cd  145  145  266
  54.3% identity in 464 nt overlap

                1230      1240      1250      1260       1270
pmsgen AGGAAGTCTCAAAGTGCCCTACAATGTTGGACCTGGCTTTAC-TGGAAACTTTTCTACAC
                                     : : : :: ::: :: :   :  :   : :
HUMTFR TATGGAAGGAGACTGTCCCTCTGACTGGAAAACAGACTCTACATGTAGGATGGTAACCTC
        1140      1150      1160      1170      1180      1190

       1280      1290      1300      1310            1320      1330
pmsgen AAAAAGTCAAGATGCACATC-CACTCT-ACCAATG------AAGTGACAAGAATTTACAA
       : ::::  :  :::   : : :::: : : :::::      ::: :: :: ::::   ::
HUMTFR AGAAAGCAAGAATGTGAAGCTCACTGTGAGCAATGTGCTGAAAGAGATAAAAATTCTTAA
        1200      1210      1220      1230      1240      1250

               1340      1350      1360      1370      1380      1390
pmsgen TGTGATAGGTACTCTCAGAGGAGCAGTGGAACCAGACAGATATGTCATTCTGGGAGGTCA
         :  : ::   : : : :::    :: ::::::::    :::::  :  : :: :  ::
HUMTFR CATCTTTGGAGTTATTAAAGGCTTTGTAGAACCAGATCACTATGTTGTAGTTGGGGCCCA
        1260      1270      1280      1290      1300      1310

               1400      1410      1420      1430       1440      1450
pmsgen CCGGGACTCATGGGTGTTTGGTGGTATTGACCCTCAGAGT-GGAGCAGCTGTTGTTCATG
         : ::  ::::::    ::: : :    :  : : : :: ::  ::::: :  :
HUMTFR GAGAGATGCATGGGGCCCTGGAGCTGCAAAATC-CGGTGTAGGCACAGCTCTCCTATTGA
        1320      1330      1340      1350       1360      1370
```

FIGURE 16-10

```
              1460      1470      1480      1490      1500
pmsgen AAATTG---TGAGGAGCTTTGGAACACTGAAAAAGGAAGGGTGGAGACCTAGAAGAACAA
       :: :::   :: :  ::  :  :   :   ::: :: X:::     :: :: ::::  :
HUMTFR AACTTGCCCAGATGTTCTCAGATATGGTCTTAAAAGATGGGTTTCAGCCCAGCAGAAGCA
         1380      1390      1400      1410      1420      1430

         1510      1520      1530      1540      1550      1560
pmsgen TTTTGTTTGCAAGCTGGGATGCAGAAGAATTTGGTCTTCTTGGTTCTACTGAGTGGGCAG
       :: : ::::: :: :::  ::: : ::: :::::     ::::: : ::::: :::  ::
HUMTFR TTATCTTTGCCAGTTGGAGTGCTGGAGACTTTGGATCGGTTGGTGCCACTGAATGGCTAG
         1440      1450      1460      1470      1480      1490

         1570      1580      1590      1600      1610      1620
pmsgen A-GGAGAATTCAAGACTCCTTCAAGAGCGTGGCGTGGCTTATATTAATGCTGACTCATCT
       : ::: :  :   : : ::: ::          :   : ::::::::::X  ::   : :
HUMTFR AGGGATACCTTTCGTC-CCTGCATTTAAAGGCTTTCACTTATATTAATCTGGATAAAGCG
         1500      1510      1520      1530      1540      1550

         1630      1640      1650      1660      1670      1680
pmsgen ATAGAAGGAAACTACACTCTGAGAGTTGATTGTACACCGCTGATGTACA-GCTTGGT-AC
       :    :: : :  ::   : :  :::  :    :  :: ::: :::: : ::::   : :
HUMTFR GTTCTTGGTACCAGCAACTTCAAGGTTTCTGCCAGCCCACTGTTGTATACGCTTATTGAG
         1560      1570      1580      1590      1600      1610
```

FIGURE 16-11

```
           1690      1700      1710      1720      1730      1740
pmsgen ACAACCTAACAAAAGAGCTGAAAAGCCCTGATGAAGGCTTTGAAGGCAAATCTCTTTATG
       : :::    :::::
HUMTFR AAAACAATGCAAAATGTGAAGCATCCGGTTACTGGGCAATTTCTATATCAGGACAGCAAC
       1620      1630      1640      1650      1660      1670
```

FIGURE 17A

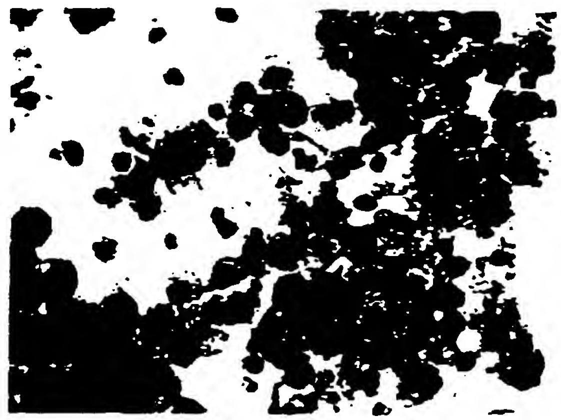


FIGURE 17B

FIGURE 17C

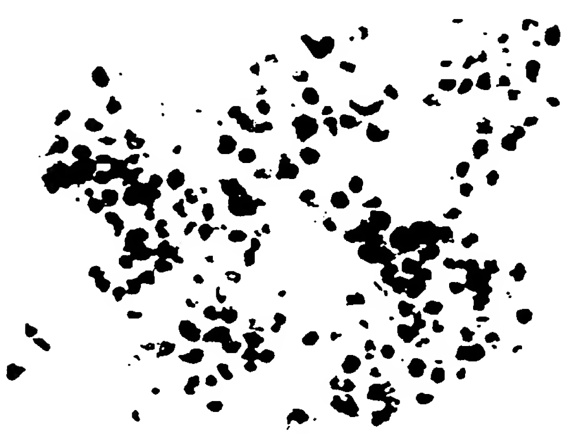


FIGURE 18

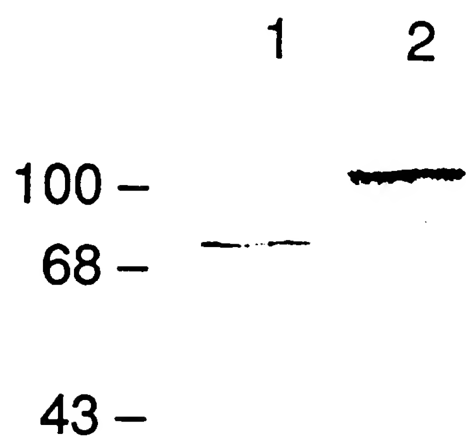


FIGURE 19

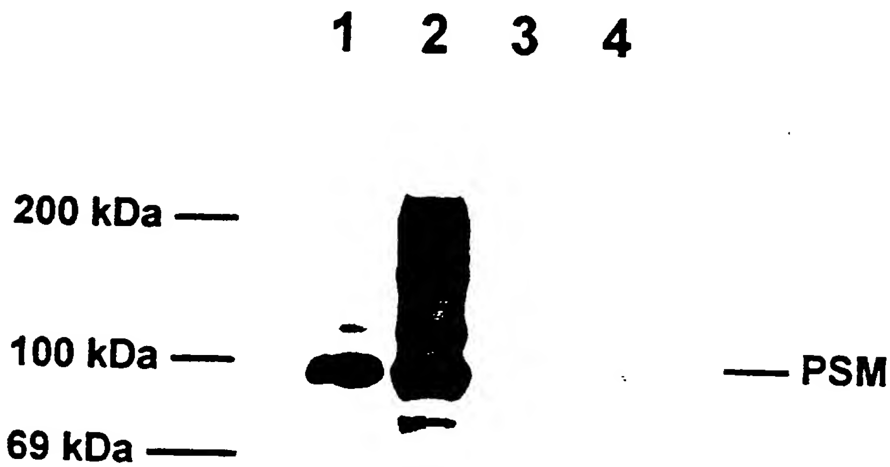


FIGURE 20

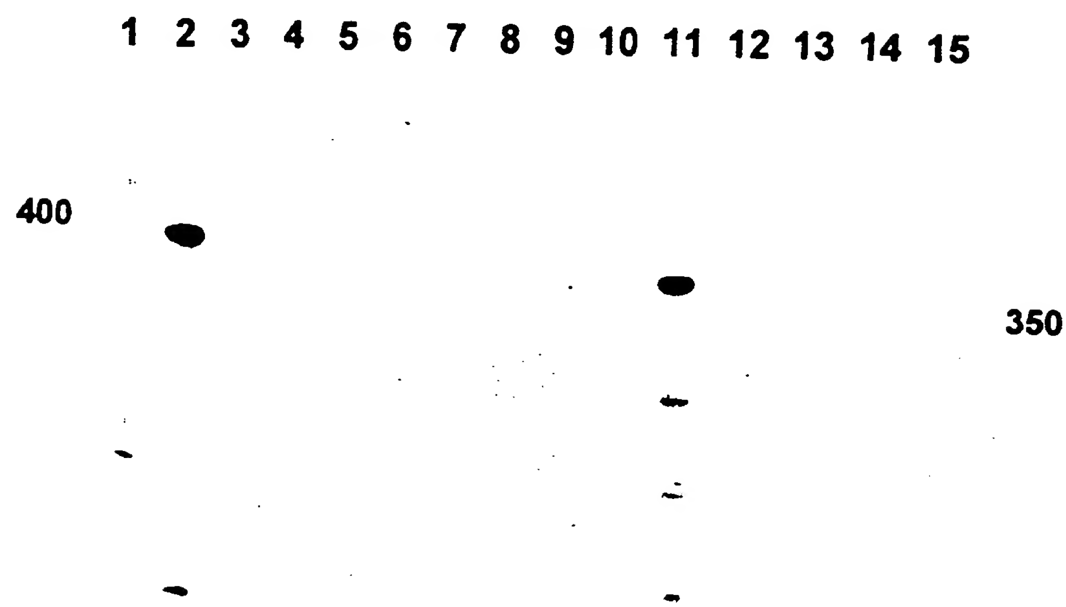


FIGURE 21

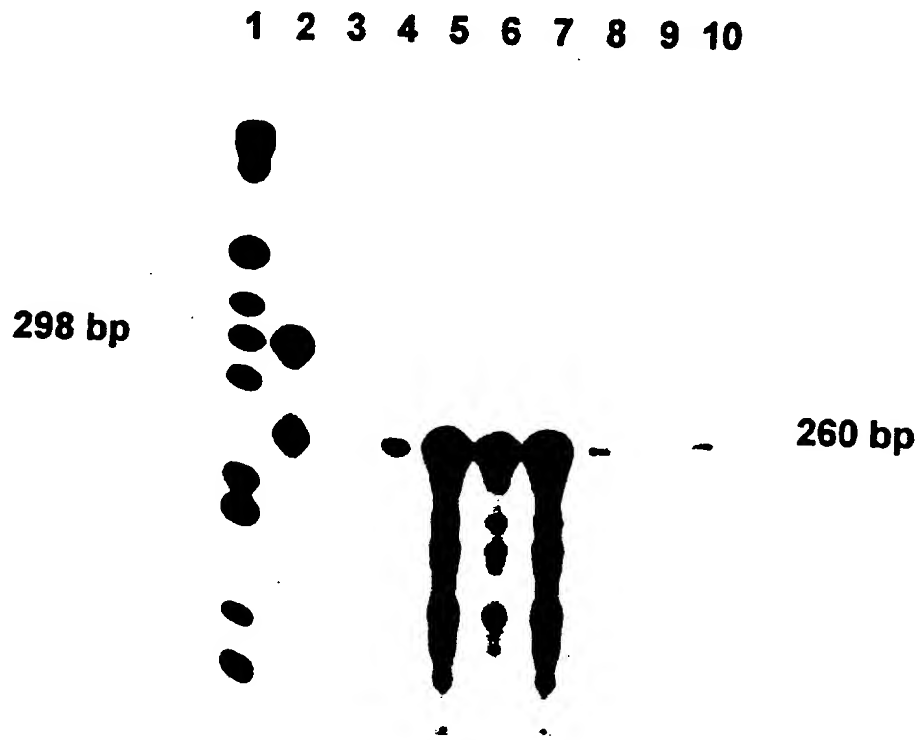


FIGURE 22

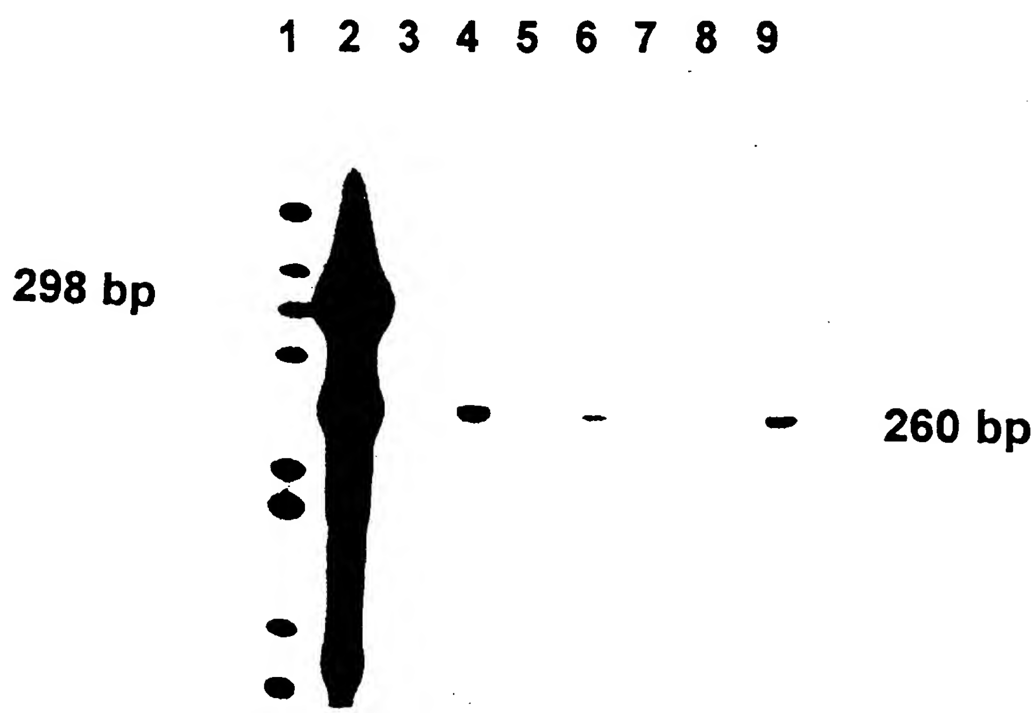


FIGURE 23

CELL LINE/TYPE	11p11.2-13 REGION	METASTATIC	PSM RNA DETECTED	PSM DNA DETECTED
LNCap			++	ND
HUMAN PROSTATE			++	ND
A9 (FIBROSARCOMA)	NO	NO	-	-
A9(11) (A9+HUM. 11)	YES	NO	-	REPEAT
AT6.1 (RAT PROSTATE)	NO	YES	-	-
AT6.1-11-c11	YES	NO	+	++
AT6.1-11-c12	NO	YES	-	-
R1564 (RAT MAMMARY)	NO	YES	-	-
R1564-11-c14	YES	YES	-	+
R1564-11-c15	YES	YES	-	REPEAT
R1564-11-c16	YES	YES	-	ND
R1564-11-c12	YES	YES	ND	+

FIGURE 24A

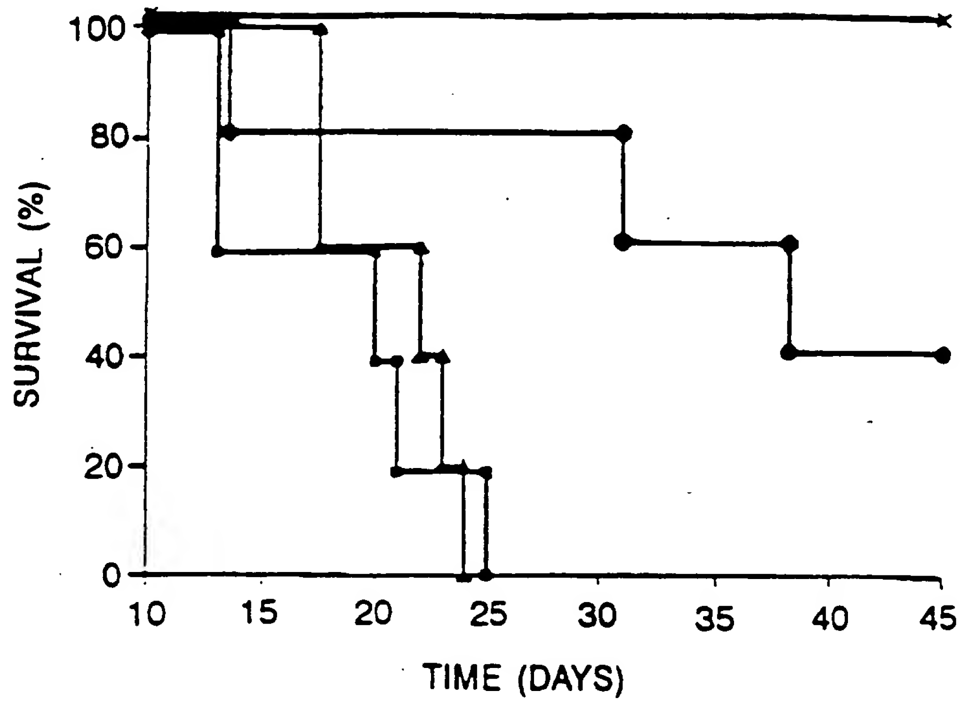


FIGURE 24B

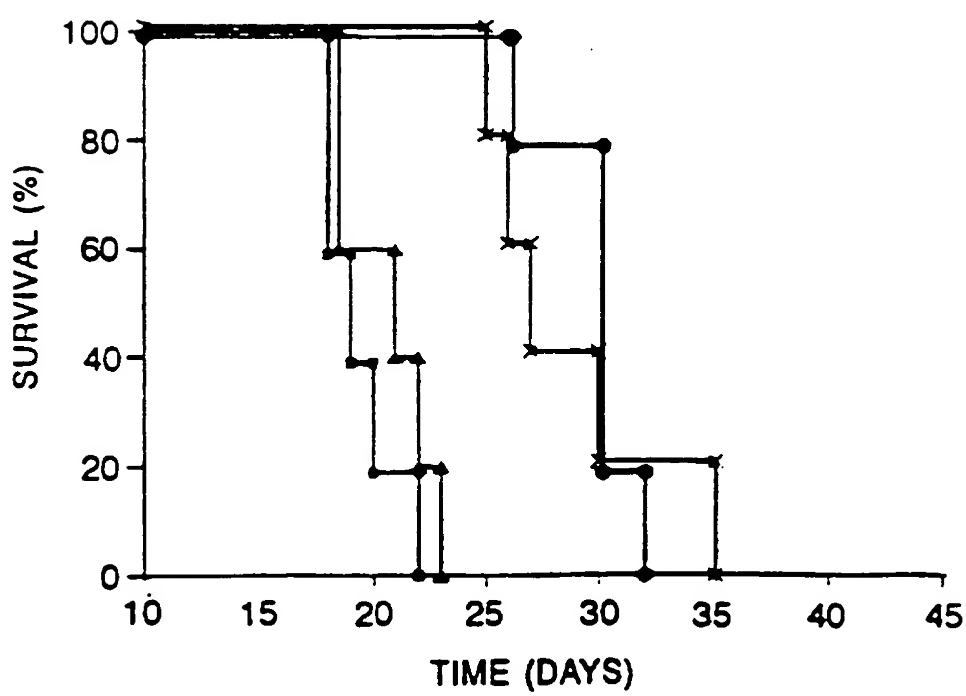


FIGURE 25A

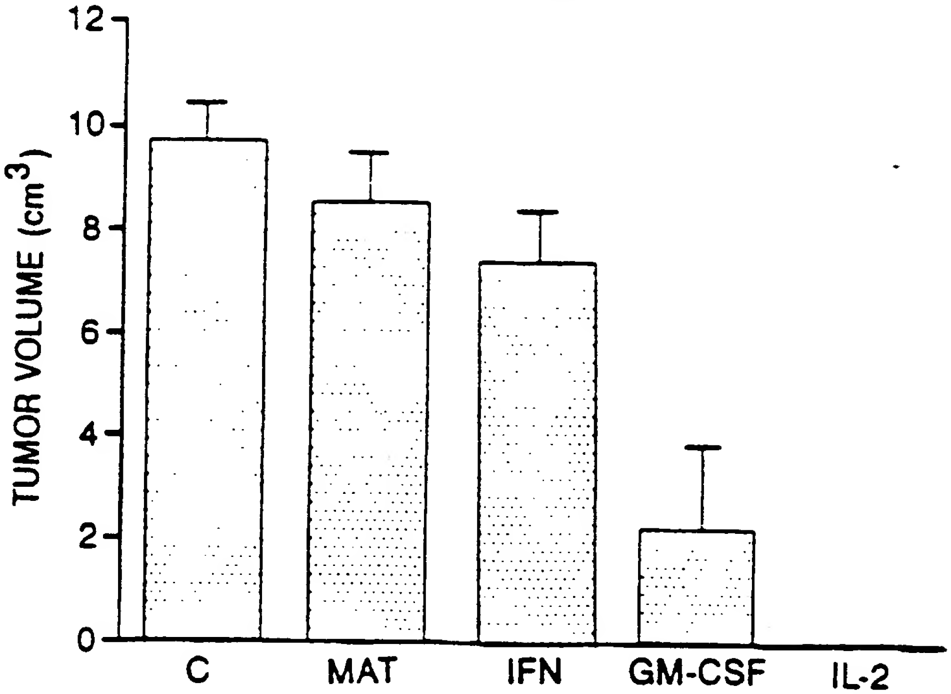


FIGURE 25B

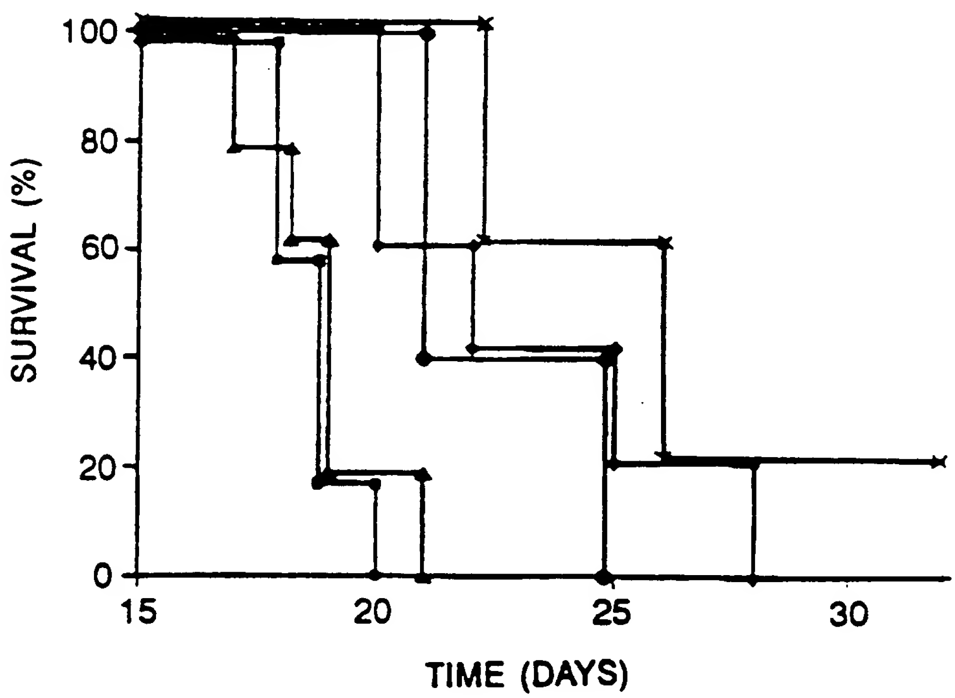


FIGURE 26

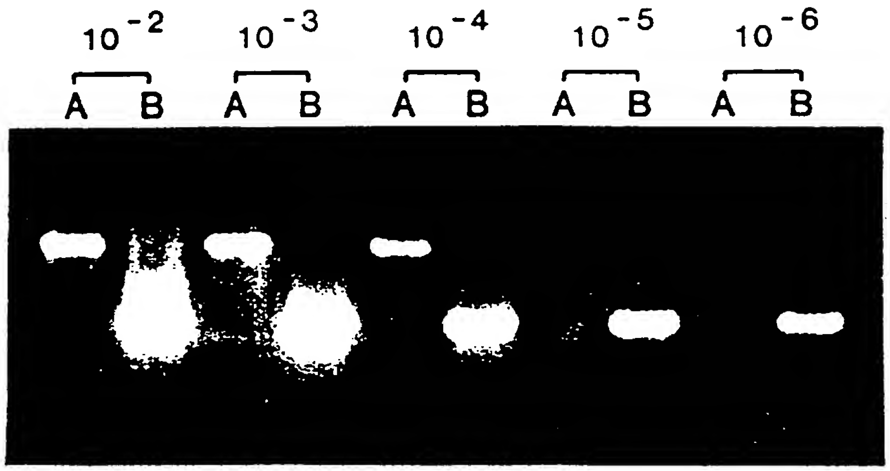


FIGURE 27

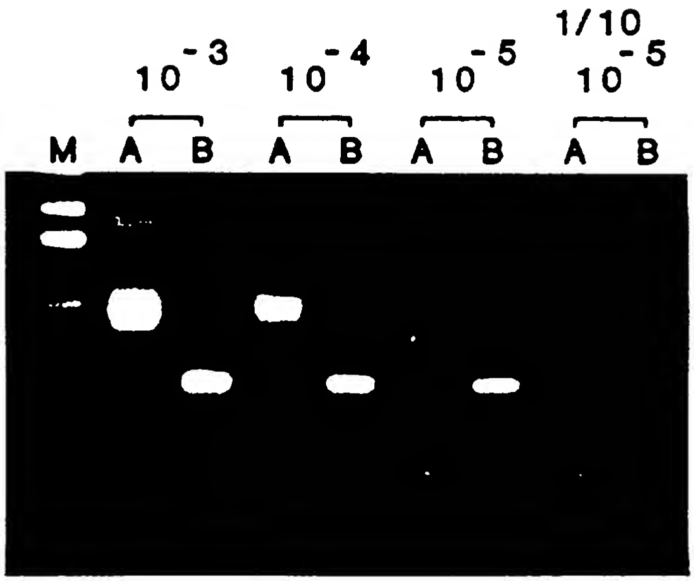


FIGURE 28

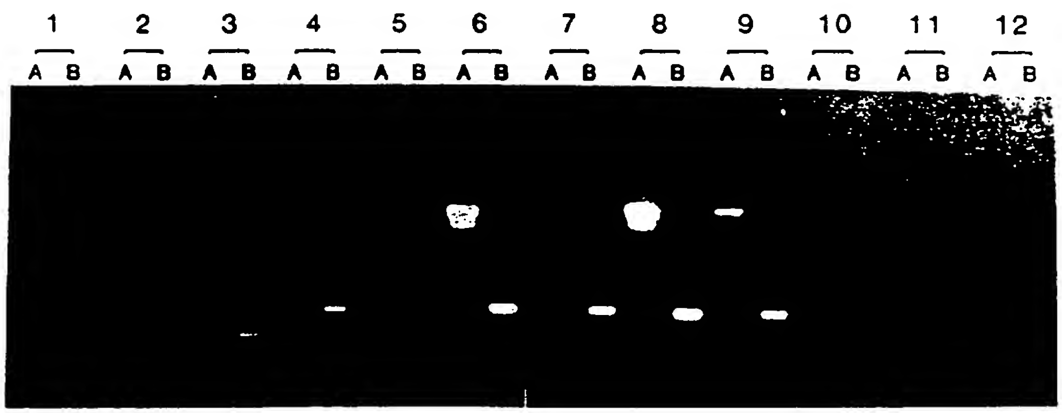


FIGURE 29

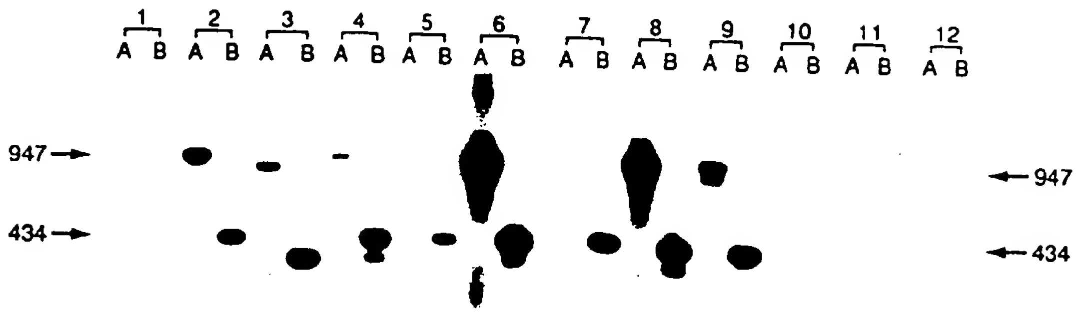

1
2
3
4
5
6
7
8
9
10
11
12
A B A B A B A B A B A B A B A B A B A B A B A B
947
434
947
434

FIGURE 30

Patient	Stage	Treatment	PSA	PAP	PSA-PCR	PSM-PCR
1	T2NxMo	None	8.9	0.7	–	+
2	T2NoMo	RRP 7/93	6.1	–	–	+
3	T2CNoMo	PLND 5/93	4.5	0.1	–	+
4	T2BNoMo	RRP 3/92	NMA	0.4	–	+
5	T3NxMo	Proscar + Flutamide	51.3	1.0	–	+
6	Recur T3	I-125 1986	54.7	1.4	–	+
7	T3ANoMo	RRP 10/92	NMA	0.3	–	+
8	T3NxMo	XRT 1987	7.5	0.1	–	–
9	T3NxMo	Proscar + Flutamide	35.4	0.7	–	–
10	D2	S/P XRT Flutamide +Emcyt	311	4.5	+	+
11	D2	RRP 4/91 Lupron 10/92 Velban + Emcyt 12/92	1534	1.4	+	+
12	T2NoMo	RRP 8/91	NMA	0.5	–	+
13	T3NoMo	RRP 1/88 Lupron + Flutamide 5/92	0.1	0.3	–	–
14	D1	PLND 1989 XRT 1989	1.6	0.4	–	–
15	D1	Proscar + Flutamide	20.8	0.5	–	–
16	T2CNoMo	RRP 4/92	0.1	0.3	–	–

FIGURE 31A

```
                 10         20         30         40         50         60
                  |          |          |          |          |          |
  1  AAGGGTGCTC CTTAGGCTGA ATGCTTGCAG ACAGGATGCT TGGTTACAGA TGGGCTGTGA
     TTCCCACGAG GAATCCGACT TACGAACGTC TGTCCTACGA ACCAATGTCT ACCCGACACT

 61  CTCGAGTGGA GTTTTATAAG GGTGCTCCTT AGGCTGAATG CTTGCAGACA GGATGCTTGG
     GAGCTCACCT CAAAATATTC CCACGAGGAA TCCGACTTAC GAACGTCTGT CCTACGAACC

121  TTACAGATGG GCTGTGAGCT GGGTGCTTGT AAGAGGATGC TTGGGTGCTA AGTGAGCCAT
     AATGTCTACC CGACACTCGA CCCACGAACA TTCTCCTACG AACCCACGAT TCACTCGGTA

181  TTGCAGTTGA CCCTATTCTT GGAACATTCA TTCCCCTCTA CCCCTGTTTC TGTTCCTGCC
     AACGTCAACT GGGATAAGAA CCTTGTAAGT AAGGGGAGAT GGGGACAAAG ACAAGGACGG

241  AGCTAAGCCC ATTTTTCATT TTTCTTTTAA CTCCTTAGCG CTCCGCAAAA CTTAATCAAT
     TCGATTCGGG TAAAAAGTAA AAAGAAAATT GAGGAATCGC GAGGCGTTTT GAATTAGTTA

301  TTCTTTAAAC CTCAGTTTTC TTATCTGTAA AAGGTAAATA ATAATACAGG GTGCAACAGA
     AAGAAATTTG GAGTCAAAAG AATAGACATT TTCCATTTAT TATTATGTCC CACGTTGTCT

361  AAAATCTAGT GTGGTTTACA TAATCACCTG TTAGAGATTT TAAATTATTT CAGGATAAGT
     TTTTAGATCA CACCAAATGT ATTAGTGGAC AATCTCTAAA ATTTAATAAA GTCCTATTCA

421  CATGATAATT AAATGAAATA ATGCACATAA AGCACATAGT GTGGTGTCCT CCATATAGAA
     GTACTATTAA TTTACTTTAT TACGTGTATT TCGTGTATCA CACCACAGGA GGTATATCTT

481  AATGCTCAGT ATATTGGTTA TTAACTACTT GTTGAAGGTT TATCTTCTCC ACTAAACTGT
     TTACGAGTCA TATAACCAAT AATTGATGAA CAACTTCCAA ATAGAAGAGG TGATTTGACA

541  AAGTTCCACA AGCCTTACAA TATGTGACAG ATATTCATTC ATTGTCTGAA TTCTTCAAAT
     TTCAAGGTGT TCGGAATGTT ATACACTGTC TATAAGTAAG TAACAGACTT AAGAAGTTTA

601  ACATCCTCTT CACCATAGCG TCTTATTAAT TGAATTATTA ATTGAATAAA TTCTATTGTT
     TGTAGGAGAA GTGGTATCGC AGAATAATTA ACTTAATAAT TAACTTATTT AAGATAACAA

661  CAAAAATCAC TTTTATATTT AACTGAAATT TGCTTACTTA TAATCACATC TAACCTTCAA
     GTTTTTAGTG AAAATATAAA TTGACTTTAA ACGAATGAAT ATTAGTGTAG ATTGGAAGTT

721  AGAAAACACA TTAACCAACT GTACTGGGTA ATGTTACTGG GTGATCCCAC GTTTTACAAA
     TCTTTTGTGT AATTGGTTGA CATGACCCAT TACAATGACC CACTAGGGTG CAAAATGTTT
```

FIGURE 31B

```
781  TGAGAAGATA TATTCTGGTA AGTTGAATAC TTAGCACCCA GGGGTAATCA GCTTGGACAG
     ACTCTTCTAT ATAAGACCAT TCAACTTATG AATCGTGGGT CCCCATTAGT CGAACCTGTC

841  GACCAGGTCC AAAGACTGTT AAGAGTCTTC TGACTCCAAA CTCAGTGCTC CCTCCAGTGC
     CTGGTCCAGG TTTCTGACAA TTCTCAGAAG ACTGAGGTTT GAGTCACGAG GGAGGTCACG

901  CACAAGCAAA CTCCATAAAG GTATCCTGTG CTGAATAGAG ACTGTAGAGT GGTACAAAGT
     GTGTTCGTTT GAGGTATTTC CATAGGACAC GACTTATCTC TGACATCTCA CCATGTTTCA

961  AAGACAGACA TTATATTAAG TCTTAGCTTT GTGACTTCGA ATGACTTACC TAATCTAGCT
     TTCTGTCTGT AATATAATTC AGAATCGAAA CACTGAAGCT TACTGAATGG ATTAGATCGA

1021 AAATTTCAGT TTTACCATGT GTAAATCAGG AAGAGTAATA GAACAAACCT TGAAGGGTCC
     TTTAAAGTCA AAATGGTACA CATTTAGTCC TTCTCATTAT CTTGTTTGGA ACTTCCCAGG

1081 CAATGGTGAT TAAATGAGGT GATGTACATA ACATGCATCA CTCATAATAA GTGCTCTTTA
     GTTACCACTA ATTTACTCCA CTACATGTAT TGTACGTAGT GAGTATTATT CACGAGAAAT

1141 AATATTAGTC ACTATTATTA GCCATCTCTG ATTAGATTTG ACAATAGGAA CATTAGGAAA
     TTATAATCAG TGATAATAAT CGGTAGAGAC TAATCTAAAC TGTTATCCTT GTAATCCTTT

1201 GATATAGTAC ATTCAGGATT TTGTTAGAAA GAGATGAAGA AATTCCCTTC CTTCCTGCCC
     CTATATCATG TAAGTCCTAA AACAATCTTT CTCTACTTCT TTAAGGGAAG GAAGGACGGG

1261 TAGGTCATCT AGGAGTTGTC ATGGTTCATT GTTGACAAAT TAATTTTCCC AAATTTTTCA
     ATCCAGTAGA TCCTCAACAG TACCAAGTAA CAACTGTTTA ATTAAAAGGG TTTAAAAAGT

1321 CTTTGCTCAG AAAGTCTACA TCGAAGCACC CAAGACTGTA CAATCTAGTC CATCTTTTTC
     GAAACGAGTC TTTCAGATGT AGCTTCGTGG GTTCTGACAT GTTAGATCAG GTAGAAAAAG

1381 CACTTAACTC ATACTGTGCT CTCCCTTTCT CAAAGCAAAC TGTTTGCTAT TCCTTGAATA
     GTGAATTGAG TATGACACGA GAGGGAAAGA GTTTCGTTTG ACAAACGATA AGGAACTTAT

1441 CACTCTGAGT TTTCTGCCTT TGCCTACTCA GCTGGCCCAT GGCCCCTAAT GTTTCTTCTC
     GTGAGACTCA AAAGACGGAA ACGGATGAGT CGACCGGGTA CCGGGGATTA CAAAGAAGAG

1501 ATCTCCACTG GGTCAAATCC TACCTGTACC TTATGGTTCT GTTAAAAGCA GTGCTTCCAT
     TAGAGGTGAC CCAGTTTAGG ATGGACATGG AATACCAAGA CAATTTTCGT CACGAAGGTA

1561 AAAGTACTCC TAGCAAATGC ACGGCCTCTC TCACGGATTA TAAGAACACA GTTTATTTTA
```

FIGURE 31C

```
     TTTCATGAGG ATCGTTTACG TGCCGGAGAG AGTGCCTAAT ATTCTTGTGT CAAATAAAAT

1621 TAAAGCATGT AGCTATTCTC TCCCTCGAAA TACGATTATT ATTATTAAGA ATTTATAGCA
     ATTTCGTACA TCGATAAGAG AGGGAGCTTT ATGCTAATAA TAATAATTCT TAAATATCGT

1681 GGGATATAAT TTTGTATGAT GATTCTTCTG GTTAATCCAA CCAAGATTGA TTTTATATCT
     CCCTATATTA AAACATACTA CTAAGAAGAC CAATTAGGTT GGTTCTAACT AAAATATAGA

1741 ATTACGTAAG ACAGTAGCCA GACATAGCCG GGATATGAAA ATAAAGTCTC TGCCTTCAAC
     TAATGCATTC TGTCATCGGT CTGTATCGGC CCTATACTTT TATTTCAGAG ACGGAAGTTG

1801 AAGTTCCAGT ATTCTTTTCT TTCCTCCCCT CCCCTCCCCT CCCTTCCCCT CCCCTTCCTT
     TTCAAGGTCA TAAGAAAAGA AAGGAGGGGA GGGGAGGGGA GGGAAGGGGA GGGGAAGGAA

1861 CCCTTTCCCT TCCCTTCCTT TCTTTCTTGA GGGAGTCTCA CTCTGTCACC AGGCTCCAGT
     GGGAAAGGGA AGGGAAGGAA AGAAAGAACT CCCTCAGAGT GAGACAGTGG TCCGAGGTCA

1921 GCAGTGGCGC TATCTTGGCT GACTGCAACC TCCGCCTCCC CGGTTCAAGC GATTCTCCTG
     CGTCACCGCG ATAGAACCGA CTGACGTTGG AGGCGGAGGG GCCAAGTTCG CTAAGAGGAC

1981 CCTCAGCCTC CTGAGTAGCT GGGACTACAG GAGCCCGCCA CCACGCCCAG CTAATTTTTG
     GGAGTCGGAG GACTCATCGA CCCTGATGTC CTCGGGCGGT GGTGCGGGTC GATTAAAAAC

2041 TATTTTTAGT AGAGATGGGG TTTCACCATG TTGGCCAGGA TGGTCTCGAT TTCTCGACTT
     ATAAAAATCA TCTCTACCCC AAAGTGGTAC AACCGGTCCT ACCAGAGCTA AAGAGCTGAA

2101 CGTGATCCGC CTGTCTGGGC CTCCCAAAGT GCTGGGATTA CAGGCGTGAG CCACCACGCC
     GCACTAGGCG GACAGACCCG GAGGGTTTCA CGACCCTAAT GTCCGCACTC GGTGGTGCGG

2161 CGGCTTTAAA AAATGGTTTT GTAATGTAAG TGGAGGATAA TACCCTACAT GTTTATTAAT
     GCCGAAATTT TTTACCAAAA CATTACATTC ACCTCCTATT ATGGGATGTA CAAATAATTA

2221 AACAATAATA TTCTTTAGGA AAAAGGGCGC GGTGGTGATT TACACTGATG ACAAGCATTC
     TTGTTATTAT AAGAAATCCT TTTTCCCGCG CCACCACTAA ATGTGACTAC TGTTCGTAAG

2281 CCGACTATGG AAAAAAAGCG CAGCTTTTTC TGCTCTGCTT TTATTCAGTA GAGTATTGTA
     GGCTGATACC TTTTTTTCGC GTCGAAAAAG ACGAGACGAA AATAAGTCAT CTCATAACAT

2341 GAGATTGTAT AGAATTTCAG AGTTGAATAA AAGTTCCTCA TAATTATAGG AGTGGAGAGA
     CTCTAACATA TCTTAAAGTC TCAACTTATT TTCAAGGAGT ATTAATATCC TCACCTCTCT
```

FIGURE 31D

```
2401 GGAGAGTCTC TTTCTTCCTT TCATTTTTAT ATTTAAGCAA GAGCTGGACA TTTTCCAAGA
     CCTCTCAGAG AAAGAAGGAA AGTAAAAATA TAAATTCGTT CTCGACCTGT AAAAGGTTCT

2461 AAGTTTTTTT TTTTTAAGGC GCCTCTCAAA AGGGGCCGGA TTTCCTTCTC CTGGAGGCAG
     TTCAAAAAAA AAAAATTCCG CGGAGAGTTT TCCCCGGCCT AAAGGAAGAG GACCTCCGTC

2521 ATGTTGCCTC TCTCTCTCGC TCGGATTGGT TCAGTGCACT CTAGAAACAC TGCTGTGGTG
     TACAACGGAG AGAGAGAGCG AGCCTAACCA AGTCACGTGA GATCTTTGTG ACGACACCAC

2581 GAGAAACTGG ACCCCAGGTC TGGAGCGAAT TCCAGCCTGC AGGGCTGATA AGCGAGGCAT
     CTCTTTGACC TGGGGTCCAG ACCTCGCTTA AGGTCGGACG TCCCGACTAT TCGCTCCGTA

2641 TAGTGAGATT GAGAGAGACT TTACCCCGCC GTGGTGGTTG GAGGGCGCGC AGTAGAGCAG
     ATCACTCTAA CTCTCTCTGA AATGGGGCGG CACCACCAAC CTCCCGCGCG TCATCTCGTC

2701 CAGCACAGGC GCGGGTCCCG GGAGGCCGGC TCTGCTCGCG CCGAGATGTG GAATCTCCTT
     GTCGTGTCCG CGCCCAGGGC CCTCCGGCCG AGACGAGCGC GGCTCTACAC CTTAGAGGAA

2761 CACGAAACCG ACTCGGCTGT GGCCACCGCG CGCCGCCCGC GCTGGCTGTG CGCTGGGGCG
     GTGCTTTGGC TGAGCCGACA CCGGTGGCGC GCGGCGGGCG CGACCGACAC GCGACCCCGC

2821 CTGGTGCTGG CGGGTGGCTT CTTTCTCCTC GGCTTCCTCT TCGGTAGGGG GGCGCCTCGC
     GACCACGACC GCCCACCGAA GAAAGAGGAG CCGAAGGAGA AGCCATCCCC CCGCGGAGCG

2881 GGAGCAAACC TCGGAGTCTT CCCCGTGGTG CCGCGGTGCT GGGACTCGCG GGTCAGCTGC
     CCTCGTTTGG AGCCTCAGAA GGGGCACCAC GGCGCCACGA CCCTGAGCGC CCAGTCGACG

2941 CGAGTGGGAT CCTGTTGCTG GTCTTCCCCA GGGGCGGCGA TTAGGGTCGG GGTAATGTGG
     GCTCACCCTA GGACAACGAC CAGAAGGGGT CCCCGCCGCT AATCCCAGCC CCATTACACC

3001 GGTGAGCACC CCTCGAG
     CCACTCGTGG GGAGCTC
```

FIGURE 32

Potential binding sites on the PSM promoter*

Site	Seq	**Location	#nt matched	
AP1	TKAGTCA	1145	7/7	
E2-RS	ACCNNNNNNGGT	1940	12/12	
		1951	12/12	
GHF	NNNTAAATNNN	580	11/11	
		753	11/11	
		1340	11/11	
		1882	11/11	
		1930	11/11	
		1979	11/11	
		2001	11/11	
		2334	11/11	
		2374	11/11	
		2591	11/11	
		2620	11/11	
		2686	11/11	
JVC repeat	GGGNGGRR	1165	8/8	
		1175	8/8	
		1180	8/8	
		1185	8/8	
		1190	8/8	
NFkB	GGGRHTYYHC	961	10/10	
uteroglobi	RYYWSGTG	250	8/8	
		921	8/8	
		1104	8/8	
IFN	AAWAANGAAAGGR590	13/13		Cell 41:509 (1985)

* the PSM promoter sequence 683XFRVS (Fig. 1) starts from the 5' end of the promoter fragment. The 3' region overlapps the previously published PSM cDNA at nt#2485,i.e. the putatative transcription start site is at nt#2485 on sequence 683XFRVS. **The number refered to in this table is in reference to sequence 683XF107 which is the complement and inverse of 683XFRVS.

FIGURE 33

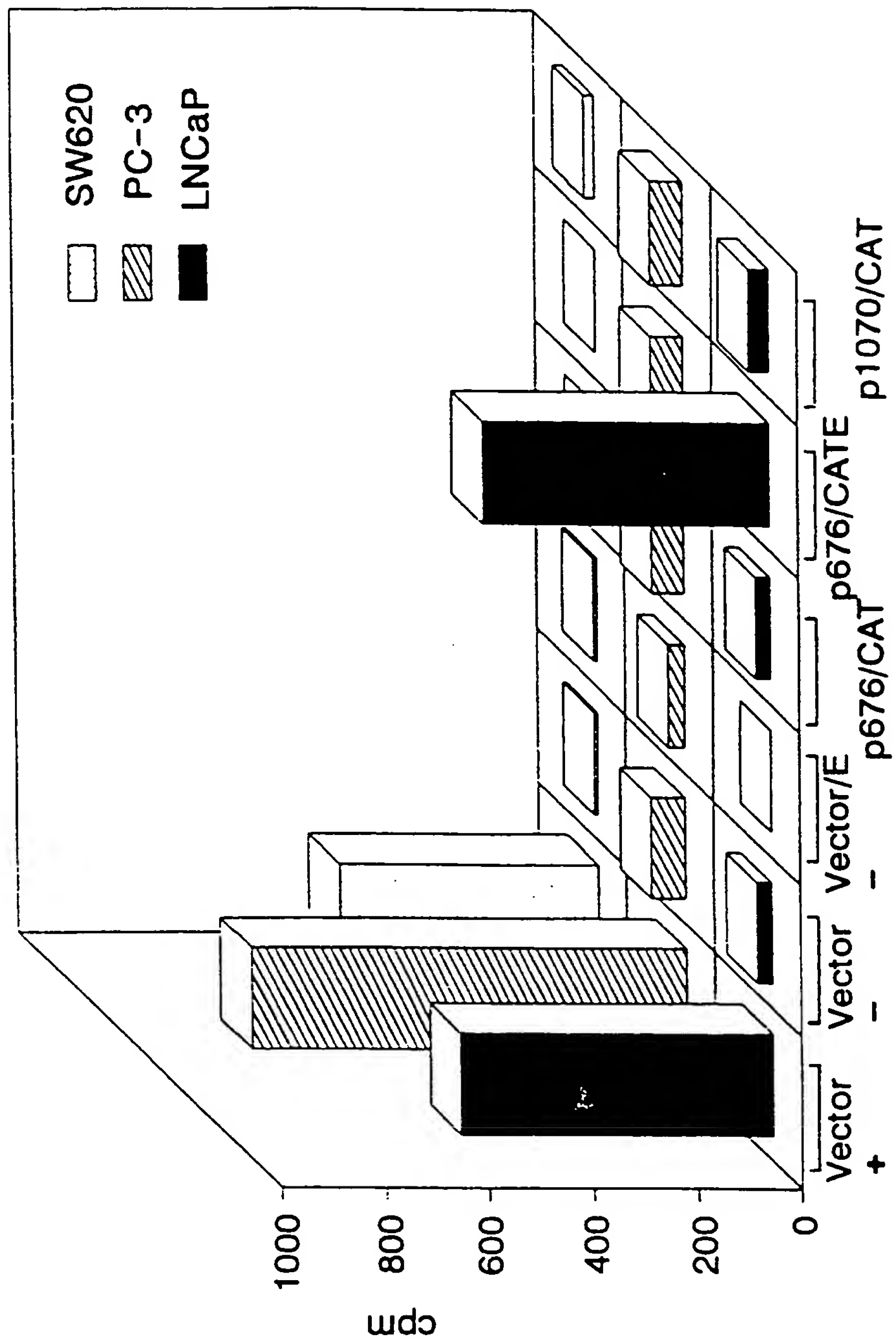

SW620
PC-3
LNCaP
cpm
1000
800
600
400
200
0
+ Vector
− Vector
− Vector/E
p676/CAT
p676/CATE
p1070/CAT

FIGURE 34

CTCAAAAGGGGCCGGATTTCCT

TCT TGGAGGCAGATGTTGCCTCTCTCTCTCGCTCGGATTGGTTCAGTGCACTCTAGAAACACTGCTGTGGTGGAGAAACT

GGACCCC AGG TCTGGAGCGAATTCCA GCCTGCAGGGCTGATAAGCGAGGCATTAGTGAGATTGAGAGAGACTTTACCC

CGCGGTGGTTGGAGGGCGCGCAGT AGAGCAGCAGCACAGGCGCGGGTCCCGGGAGGCCGGCTCTGCTCGCGCCGAG

ATG TGG AAT CTC CTT CAC GAA ACC GAC TCG GCT GTG GCC ACC GCG CGC CGC CCG CGC TGG CTG

Met Trp Asn Leu Leu His Glu Thr Asp Ser Ala Val Ala Ala Ala Arg Arg Pro Arg Trp Leu

TGC GCT GGG GCG CTG GTG CTG GCG GGT GGC TTC TTT CTC CTC GGC TTC CTC TTC GGA TGG TTT

Cys Ala Gly Ala Leu Val Leu Ala Gly Gly Phe Phe Leu Leu Gly Phe Leu Phe Gly Trp Phe

ATA AAA TCC TCC AAT GAA GCT ACT AAC ATT ACT CCA AAG CAT AAT ATG AAA GCA TTT TTG GAT GAA

Ile Lys Ser Ser Asn Glu Ala Thr Asn Ile Thr Pro Lys His Asn Met Lys Ala Phe Leu Asp Glu

*

TGG AAA GCT GAG AAC ATC AAG AAG TTC TTA TAT AAT TTT ACA CAG ATA CCA CAT TTA GCA GGA ACA

Leu Lys Ala Glu Asn Ile Lys Lys Phe Leu Tyr Asn Phe Thr Gln Ile Pro His Leu Ala Gly Thr

FIGURE 35

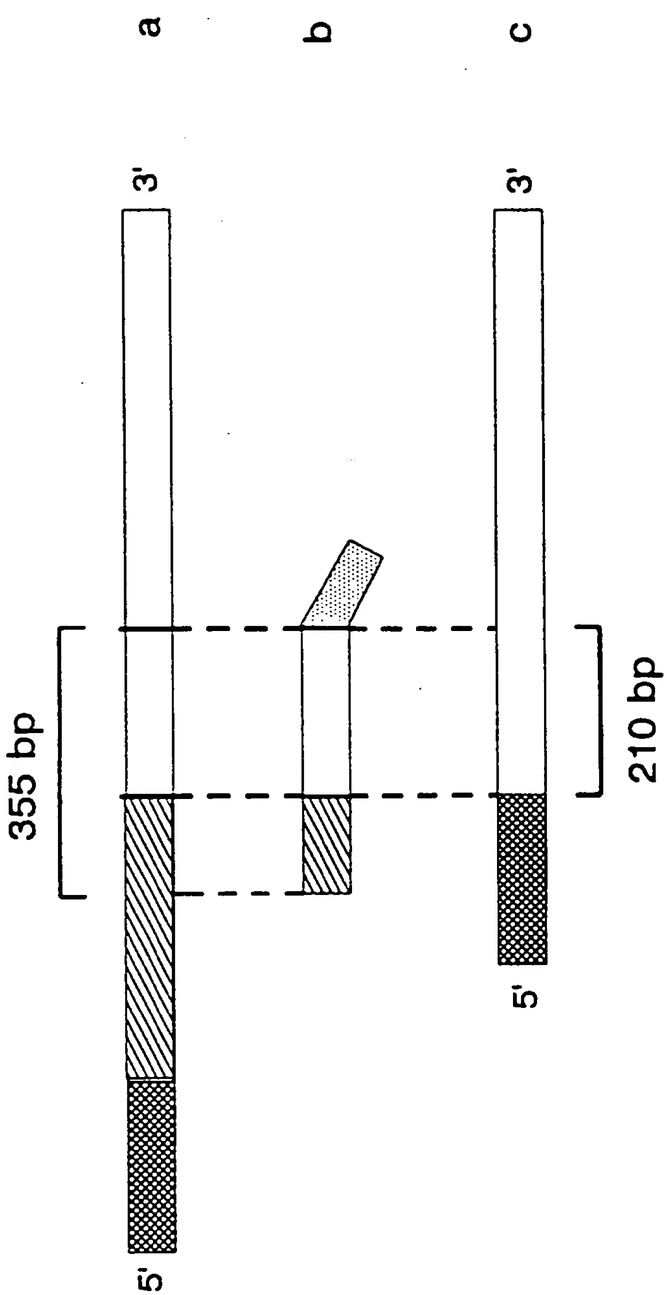


FIGURE 36

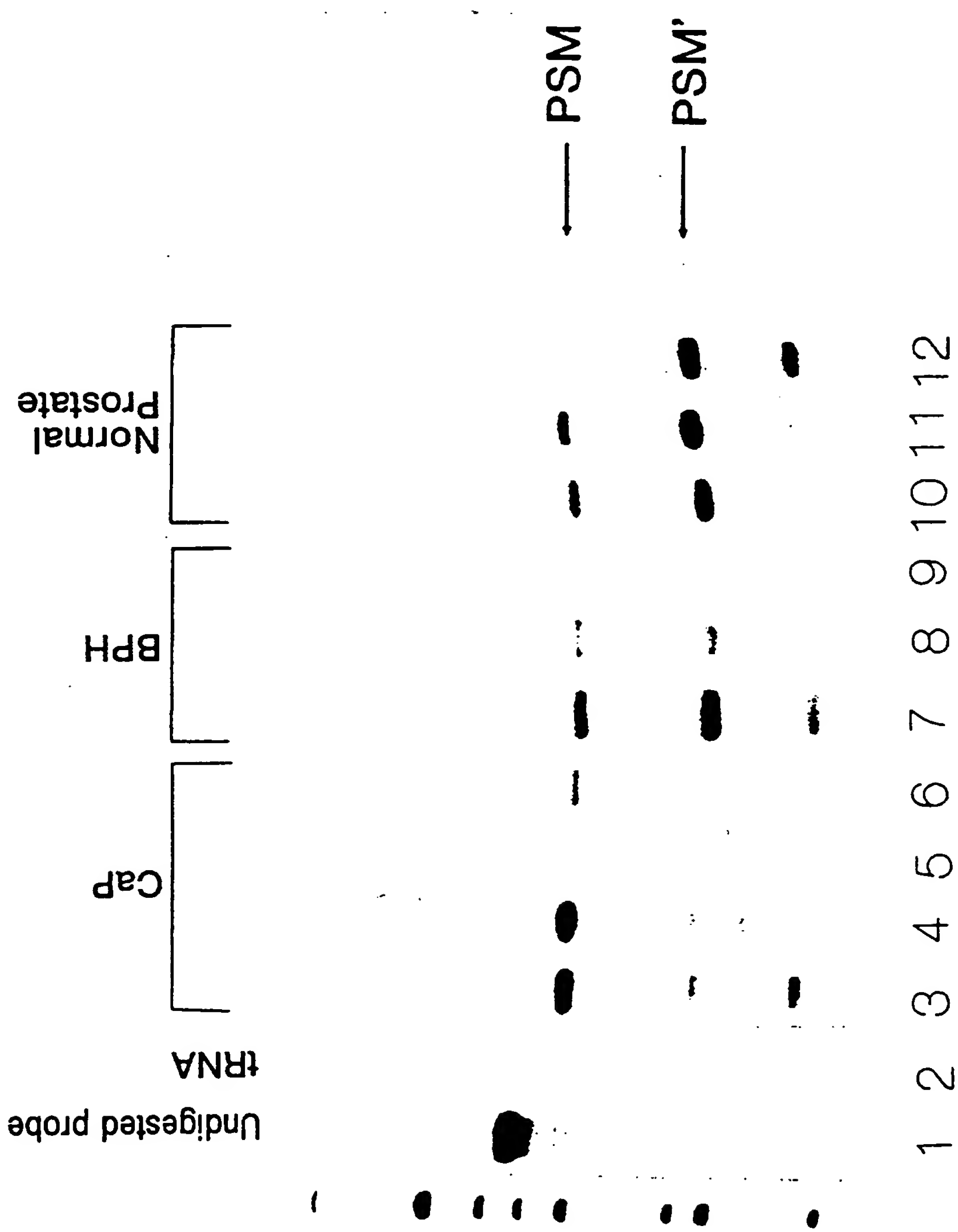

PSM
PSM'
Normal Prostate
BPH
CaP
tRNA
Undigested probe
1 2 3 4 5 6 7 8 9 10 11 12

FIGURE 37

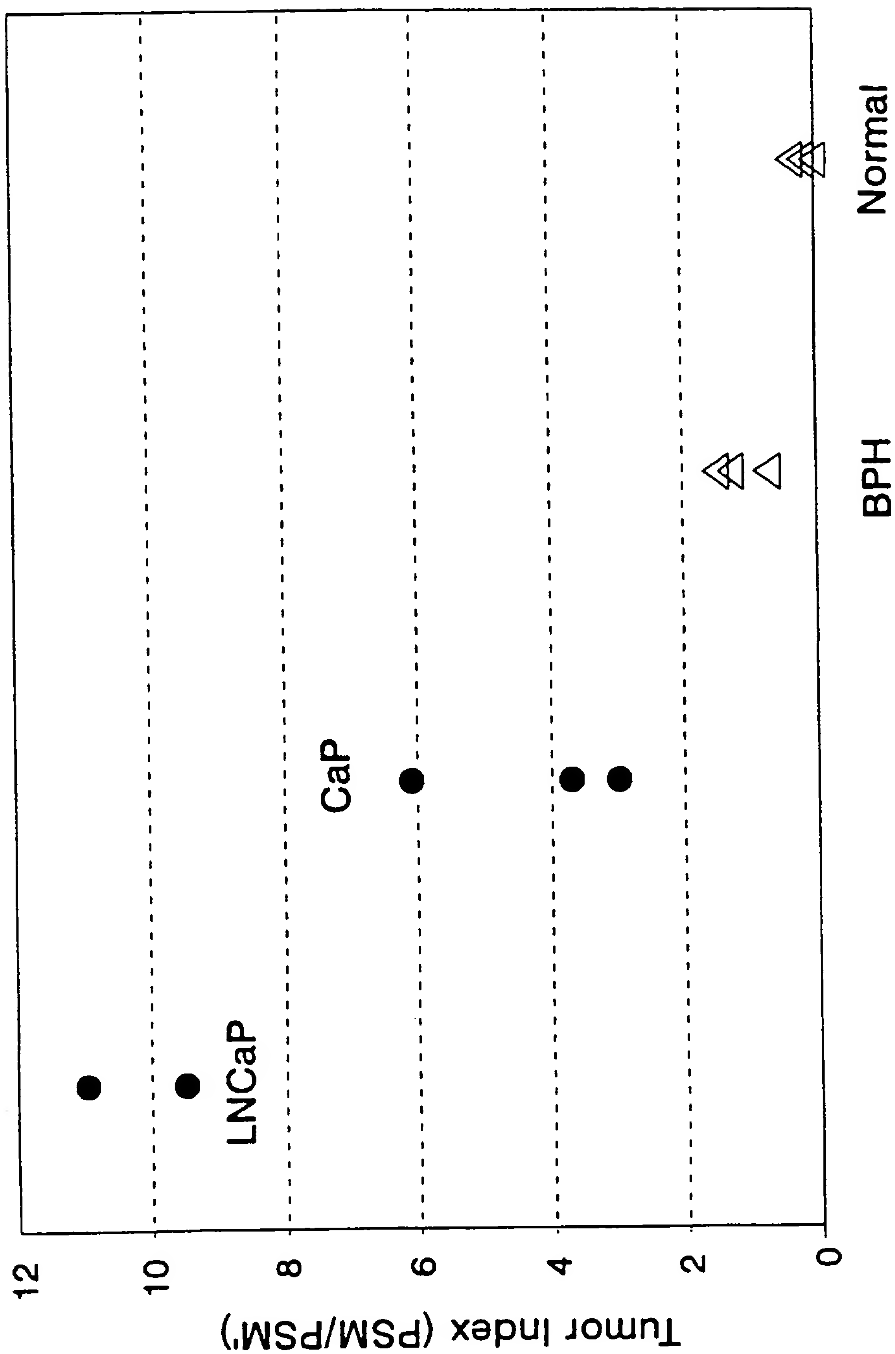


FIGURE 38

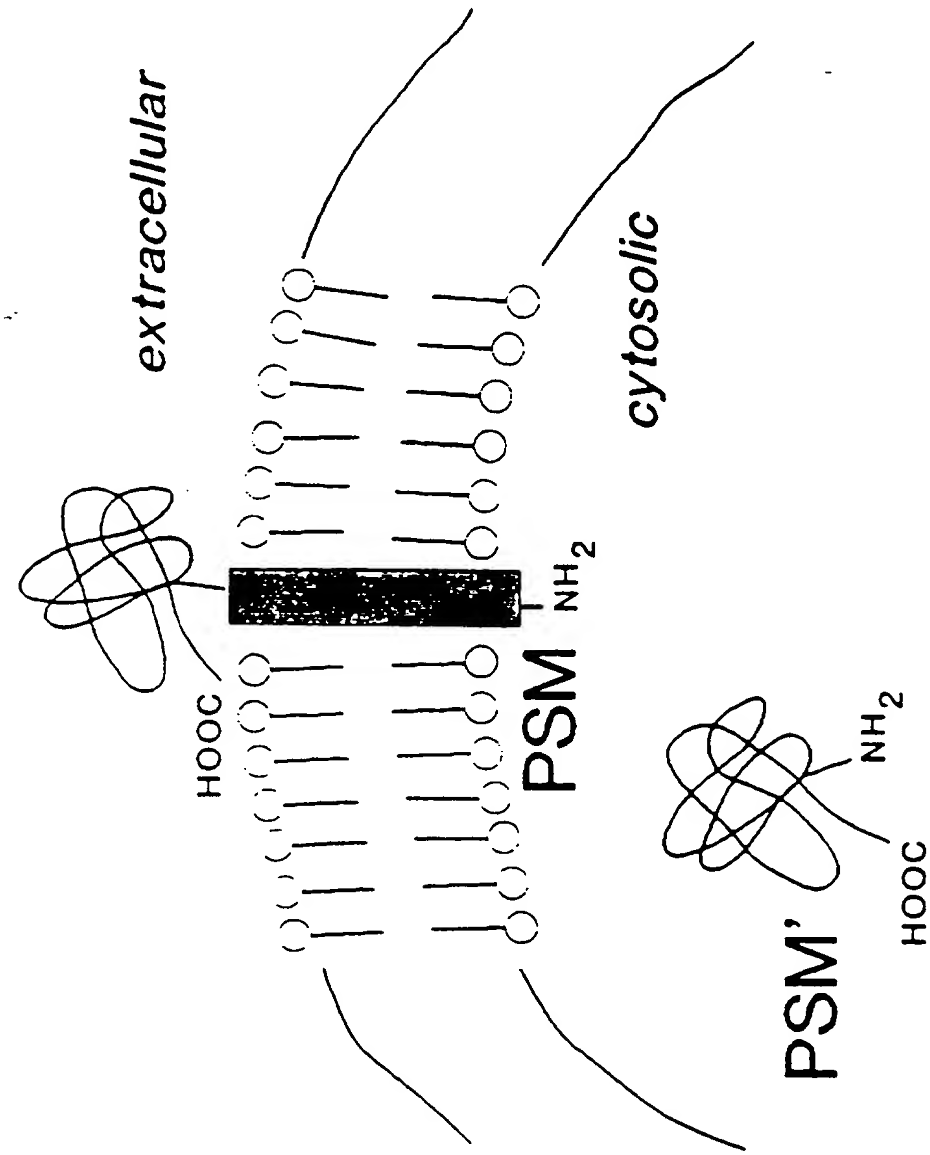


FIGURE 39

```
              10         20         30         40         50         60
               |          |          |          |          |          |
  1 TTTGCAGACT TGACCAACTT TCTAAGAAAA GCAGAACCAC ACAGGCAAGC TCAGACTCTT
    AAACGTCTGA ACTGGTTGAA AGATTCTTTT CGTCTTGGTG TGTCCGTTCG AGTCTGAGAA

 61 TTATTAAATT CCAGTTTTGA CTTTGCCACT TCTTAGTGGC CTTGAACAAG TTACCGAGTC
    AATAATTTAA GGTCAAAACT GAAACGGTGA AGAATCACCG GAACTTGTTC AATGGCTCAG

121 CTCTCAGCGT TAGTTACCCT ATTTTAATGA TGAGGATAAT ATTATCTGCC CAAATTATTG
    GAGAGTCGCA ATCAATGGGA TAAAATTACT ACTCCTATTA TAATAGACGG GTTTAATAAC

181 GTATAGTAAA TATATAGCAT GTAAATCTCC TAGCAGAGTA CTGGGATTTC GCCACTTTAT
    CATATCATTT ATATATCGTA CATTTAGAGG ATCGTCTCAT GACCCTAAAG CGGTGAAATA

241 TTCTTCTTTA CCAAGATACT CCTATTGGAC TTAATACACA GGACTAGTCT AAGGTATCAC
    AAGAAGAAAT GGTTCTATGA GGATAACCTG AATTATGTGT CCTGATCAGA TTCCATAGTG

301 CAGGTAGTCC ACTCCTGCTC GGAATCTGAC CCGGGATTAG AGTAGGGCAT GGACCAGATG
    GTCCATCAGG TGAGGACGAG CCTTAGACTG GGCCCTAATC TCATCCCGTA CCTGGTCTAC

361 GGTTTAAACA AATTCAATAT CTTCCACTAG CTTCACCTTG GGGTTGTAAA AGTTTTTGAA
    CCAAATTTGT TTAAGTTATA GAAGGTGATC GAAGTGGAAC CCCAACATTT TCAAAAACTT

421 GGACACACTG TGCTCATAAC AATCTTCATC TCTTAAAAGG ATTTTATTCT TCCTGGTATC
    CCTGTGTGAC ACGAGTATTG TTAGAAGTAG AGAATTTTCC TAAAATAAGA AGGACCATAG

481 CTCACTCTCA TCCCTTGTAT TCCGTGCTCA GTGGCTGACA CAGAAGAGTT CTTTATNNNN
    GAGTGAGAGT AGGGAACATA AGGCACGAGT CACCGACTGT GTCTTCTCAA GAAATANNNN

541 NNNNNNNNNN CATCCTGTTC ATTTTTCAGA TCTCAGTTCA AGCATCTCGT CCTCAGTGTG
    NNNNNNNNNN GTAGGACAAG TAAAAAGTCT AGAGTCAAGT TCGTAGAGCA GGAGTCACAC

601 GTGTTNNCTG ATCCCTCACT CTAATCCAAG TCTTTCTGTT TTATGCACAG GTTGGAATCT
    CACAANNGAC TAGGGAGTGA GATTAGGTTC AGAAAGACAA AATACGTGTC CAACCTTAGA

661 TATTTCCGTT TGCGNNCCAA TCNAATNGTA TTTAATATGC ATGTATATAT GTATGTGCAT
    ATAAAGGCAA ACGCNNGGTT AGNTTANCAT AAATTATACG TACATATATA CATACACGTA

721 TTGTATGCTA NGCGATTAAG AACTAGAATA ATTAATAATT GGAAGTCTAG AAGTGG
    AACATACGAT NCGCTAATTC TTGATCTTAT TAATTATTAA CCTTCAGATC TTCACC
```

FIGURE 40A

```
              10         20         30         40         50         60
               |          |          |          |          |          |
  1 TGAAAAATAC ATCAAAAATA GGCATGAGAT ACGAGCCTAT AGATAGGACT TATTTTTTAT
    ACTTTTTATG TAGTTTTTAT CCGTACTCTA TGCTCGGATA TCTATCCTGA ATAAAAAATA

 61 TATTGTTGTA TGTATTATTT GTAAAACACA AATTATCAAT ATTACCTCTG ACATTAGGTG
    ATAACAACAT ACATAATAAA CATTTTGTGT TTAATAGTTA TAATGGAGAC TGTAATCCAC

121 AGATATTCTG AATTTTAATT TCTCTTGCCT ACTTTCACTG AAAAAGAGTC ATGCAAACAG
    TCTATAAGAC TTAAAATTAA AGAGAACGGA TGAAAGTGAC TTTTTCTCAG TACGTTTGTC

181 ATTTTTAAGT TGCAAACCAA TTGCAAAATA TTTTTTTATC CAACTTCAAT GATAGGTATT
    TAAAAATTCA ACGTTTGGTT AACGTTTTAT AAAAAAATAG GTTGAAGTTA CTATCCATAA

241 GCTGTTAATT CTAAGATATG CATTAATTGT TTCAACTAAT GGGTGTCAAA CGAGATGTTC
    CGACAATTAA GATTCTATAC GTAATTAACA AAGTTGATTA CCCACAGTTT GCTCTACAAG

301 TGAAAATGAA GGCAAAAAGG AGATCCACCT TCTACTTTCA TAAAGTTTCT ATCTTCCTCT
    ACTTTTACTT CCGTTTTTCC TCTAGGTGGA AGATGAAAGT ATTTCAAAGA TAGAAGGAGA

361 GCTGACTCAA ATAAGCATTT AATACATTTT ATAACGAATT AATTATGAAT ATATTTCAAA
    CGACTGAGTT TATTCGTAAA TTATGTAAAA TATTGCTTAA TTAATACTTA TATAAAGTTT

421 TAAATAAATT ATTTCCAAGT GTTGAAGGAA ATTCAGACTT CTAATTTGCT CTGATTCTGA
    ATTTATTTAA TAAAGGTTCA CAACTTCCTT TAAGTCTGAA GATTAAACGA GACTAAGACT

481 AACTAAAACA AATGCTCTGT GAGAGTTTGC GTTTCCAGTG AAGTAGCGTG AGAAATCCAA
    TTGATTTTGT TTACGAGACA CTCTCAAACG CAAAGGTCAC TTCATCGCAC TCTTTAGGTT

541 GTCAGACAGC TACATGAAAC TACATTTACC AGCTCTCTGC CAGACACCAG TGCACGATAG
    CAGTCTGTCG ATGTACTTTG ATGTAAATGG TCGAGAGACG GTCTGTGGTC ACGTGCTATC

601 CGCAGAACAT GTAGCTAGAT CTCAGTCATA GCTNNNNNNN NNNNNNNNNN AGACCTTGCA
    GCGTCTTGTA CATCGATCTA GAGTCAGTAT CGANNNNNNN NNNNNNNNNN TCTGGAACGT

661 GTTGGCTTTT AACCTGAAGG AGATAAGGCA AGATTCCAGG GTTTATTTAG AGAAATTACA
    CAACCGAAAA TTGGACTTCC TCTATTCCGT TCTAAGGTCC CAAATAAATC TCTTTAATGT

721 GGATCTGGGA ATAAAGTAGT TACAAAATTA GTCCCCAACC AGCTTTCATG GAGCTTTCAA
    CCTAGACCCT TATTTCATCA ATGTTTTAAT CAGGGGTTGG TCGAAAGTAC CTCGAAAGTT
```

FIGURE 40B

```
 781 TTATTAATTA TTCTAGTTCT TAATCGCATG CATACAATGC ACATACATAT ATACATGCAT
     AATAATTAAT AAGATCAAGA ATTAGCGTAC GTATGTTACG TGTATGTATA TATGTACGTA

 841 ATTAAAATAC ATGATTGGAC GCAAACGGAA ATAAGATTCC ACCTGTGCAT AAAACAGAAA
     TAATTTTATG TACTAACCTG CGTTTGCCTT TATTCTAAGG TGGACACGTA TTTTGTCTTT

 901 GACTTGGTTA GAGTGAGGGA TCAGGAAACA CCACACTGAG GACGAGATGN NNNNNNNNNN
     CTGAACCAAT CTCACTCCCT AGTCCTTTGT GGTGTGACTC CTGCTCTACN NNNNNNNNNN

 961 NTAGTGGGTG GGGGGCGGAC ATCAATAAAG AACTCTTCTG TGTCAGCCAC TGAGCACGGA
     NATCACCCAC CCCCCGCCTG TAGTTATTTC TTGAGAAGAC ACAGTCGGTG ACTCGTGCCT

1021 ATAAAGGGAT GAGAGTGAGG GCAANTACCA GAAGAATAAA ATCCTTTTAA GAGATGAAGA
     TATTTCCCTA CTCTCACTCC CGTTNATGGT CTTCTTATTT TAGGAAAATT CTCTACTTCT

1081 TTGTTATGAG CACAGTGTGT GGNTTCAAAA ATCTTTTAAC AACCCCAAGG TGAAGCTAGT
     AACAATACTC GTGTCACACA CCNAAGTTTT TAGAAAATTG TTGGGGTTCC ACTTCGATCA

1141 TGGAAGATAT TTGAATTTGT TTAAACCCAT CTGGTCCTAG CCCTATTCTT TGAATCCGAA
     ACCTTCTATA AACTTAAACA AATTTGGGTA GACCAGGATC GGGATAAGAA ACTTAGGCTT

1201 GAGGTCAAGA ATTCCGAGCA GAGTGGACTA CCTGTGATAC CTTAGACTAG TCCTGTGTAT
     CTCCAGTTCT TAAGGCTCGT CTCACCTGAT GGACACTATG GAATCTGATC AGGACACATA

1261 TCAAGTCCAA TGAGAGTATC TGTAAGAGAA TAAGTGCGAA ATCCAGATCT
     AGTTCAGGTT ACTCTCATAG ACATTCTCTT ATTCACGCTT TAGGTCTAGA
```

FIGURE 41

```
                10         20         30         40         50         60
                 |          |          |          |          |          |
  1 GGATTCTGTT GAGCCCTAGC TCATTATGAT GTCCTGTTGT CCTACCCAAA TAAGACTCAT
    CCTAAGACAA CTCGGGATCG AGTAATACTA CAGGACAACA GGATGGGTTT ATTCTGAGTA

 61 CCCAACTACA TCTCAATAAT TAATGAAGAT GGAAATGAGG TAAAAAATAA ATAAATAAAT
    GGGTTGATGT AGAGTTATTA ATTACTTCTA CCTTTACTCC ATTTTTTATT TATTTATTTA

121 AAAAGAAACA TTCCCCCCCA TTTATTATTT TTTCAAATAC CTTCTATGAA ATAATGTTCT
    TTTTCTTTGT AAGGGGGGGT AAATAATAAA AAAGTTTATG GAAGATACTT TATTACAAGA

181 ATCCCTCTCT AAATATTAAT AGAAATCAAT ATTATTGGAA CTGTGAATAC CTTTAATATC
    TAGGGAGAGA TTTATAATTA TCTTTAGTTA TAATAACCTT GACACTTATG GAAATTATAG

241 TCATTATCCG GTGTCAACTA CTTTCCTATG ATGTTGAGTT ACTGGGTTTA GAAGTCGGGA
    AGTAATAGGC CACAGTTGAT GAAAGGATAC TACAACTCAA TGACCCAAAT CTTCAGCCCT

301 AATAATGCTG TAAANNNNNN AGTTAGTCTA CACACCAATA TCAAATATGA TATACTTGTA
    TTATTACGAC ATTTNNNNNN TCAATCAGAT GTGTGGTTAT AGTTTATACT ATATGAACAT

361 AACCTCCAAG CATAAAAAGA GATACTTTAT AAAAGAGGTT CTTTTTTTCT TTTTTTTTTT
    TTGGAGGTTC GTATTTTTCT CTATGAAATA TTTTCTCCAA GAAAAAAAGA AAAAAAAAAA

421 TCCAGATGGA GTTTCACTCC TGTCAGGCAG GCNGAGTGCA GTGGTGCCAT CTCGGCTCAC
    AGGTCTACCT CAAAGTGAGG ACAGTCCGTC CGNCTCACGT CACCACGGTA GAGCCGAGTG

481 TGCAACCTCC ACCTCCCATG TTCAAGGGAT TCTCCTTCCT CAGTCTCCTG AGTAGCTGGG
    ACGTTGGAGG TGGAGGGTAC AAGTTCCCTA AGAGGAAGGA GTCAGAGGAC TCATCGACCC

541 ATTACAGGTG TGCACCACCA CACCCAGCTA ATTTTTGTAT TTTTAATAGA GACAGGGTTT
    TAATGTCCAC ACGTGGTGGT GTGGGTCGAT TAAAAACATA AAAATTATCT CTGTCCCAAA

601 CGATCGATGT TGGCCAGGCT AGTCTCGAAC TCCTGACCTC TAGGTGATCC ACCCGCTCAG
    GCTAGCTACA ACCGGTCCGA TCAGAGCTTG AGGACTGGAG ATCCACTAGG TGGGCGAGTC

661 CTCCCAAAGT TGTAGAATTA CACGTGTGAG GCACTGCGCC TTGCCAGGAG ATACATTTTT
    GAGGGTTTCA ACATCTTAAT GTGCACACTC CGTGACGCGG AACGGTCCTC TATGTAAAAA

721 GATAGGTTTA ATTTATAAAG ACACTGCACA GATTTGAGTT GCTGGGAAAT GCACGGATTC
    CTATCCAAAT TAAATATTTC TGTGACGTGT CTAAACTCAA CGACCCTTTA CGTGCCTAAG

781 CAGTATGCA
    GTCATACGT
```

FIGURE 42

```
           10         20         30         40         50         60
            |          |          |          |          |          |
  1 AATCAAAATA AAACAGTTAA AGTTTGATTA CTATAATCAA ACACAAAAAA AATGAATATT
    TTAGTTTTAT TTTGTCAATT TCAAACTAAT GATATTAGTT TGTGTTTTTT TTACTTATAA

 61 ATCTTTTATG TCAGTAGAGG CTGAATGAAT CCTTCAGGAT TTTGATGATA GTATCAGATA
    TAGAAAATAC AGTCATCTCC GACTTACTTA GGAAGTCCTA AAACTACTAT CATAGTCTAT

121 CCCAGCACTA TGCTAGAAGT TGTGAAGAAT TCACGAGATG AATAAATCAC AGATTCTGTC
    GGGTCGTGAT ACGATCTTCA ACACTTCTTA AGTGCTCTAC TTATTTAGTG TCTAAGACAG

181 CTCAAAATGG TTAGATCTAT TCAGGAAACA AAGCTAAAAA AACCCCACCA ATAACTAAAA
    GAGTTTTACC AATCTAGATA AGTCCTTTGT TTCGATTTTT TTGGGGTGGT TATTGATTTT

241 ATCAACCAAA TGAAAAACAA CAATCATAAA ATAAGTAAGT ACCTATAGAA AGAAAAGCTC
    TAGTTGGTTT ACTTTTTGTT GTTAGTATTT TATTCATTCA TGGATATCTT TCTTTTCGAG

301 AGAGGAGGTA AAAAGAATCT CCTTAAAAGG AATACTATAT ACTGTAAAAC TGTGACTGAT
    TCTCCTCCAT TTTTCTTAGA GGAATTTTCC TTATGATATA TGACATTTTG ACACTGACTA

361 AGAAGGAA
    TCTTCCTT
```

FIGURE 43A

```
                10         20         30         40         50         60
                 |          |          |          |          |          |
  1  TATGGGAAAG TTTTCAGAGG AAATAAGGTA AGGGAAAAGT TATCTCTTTT TTTCTCTCCC
     ATACCCTTTC AAAAGTCTCC TTTATTCCAT TCCCTTTTCA ATAGAGAAAA AAAGAGAGGG

 61  CCAATGTAAA AAGTTATAGT GGGTTTTACA TGTGTAGAAT CATTTTCTTA AAACTTTATG
     GGTTACATTT TTCAATATCA CCCAAAATGT ACACATCTTA GTAAAAGAAT TTTGAAATAC

121  AATACCATTA TTTTCTTGTA TTCTGTGACA TGCCACCTTA CAGAGAGGAC ACATTTACTA
     TTATGGTAAT AAAAGAACAT AAGACACTGT ACGGTGGAAT GTCTCTCCTG TGTAAATGAT

181  GGTTATATCC CGGGGTTAAA TTCGAGCATT GGAATTTGGC CAGTGTAGAT GTTTAGAGTG
     CCAATATAGG GCCCCAATTT AAGCTCGTAA CCTTAAACCG GTCACATCTA CAAATCTCAC

241  AACAGAACAA TTTTTCTGTG CTTACAGGTT ATGGCTGTGG CGTACAAGAA GCATGCACTG
     TTGTCTTGTT AAAAAGACAC GAATGTCCAA TACCGACACC GCATGTTCTT CGTACGTGAC

301  GGTTTATTAT TAACTTTCAG TATCTTTGTT TTAAATATTT TCTACAAAAA TGTTTACTAA
     CCAAATAATA ATTGAAAGTC ATAGAAACAA AATTTATAAA AGATGTTTTT ACAAATGATT

361  ATTAAATTGT AGTATGAATT GTTATAAATA ATGAGGGAAA CATTTACACA TAGCAAATTT
     TAATTTAACA TCATACTTAA CAATATTTAT TACTCCCTTT GTAAATGTGT ATCGTTTAAA

421  AAAAATTACT GTCATTTGAT TTGTTAATAT ATTTTTCTCT TTAGTGGGAA ATTAAATTAA
     TTTTTAATGA CAGTAAACTA AACAATTATA TAAAAAGAGA AATCACCCTT TAATTTAATT

481  AAAATTCCTT TCGACTGTCA GACAATAGGA TTGCTGTGGT CTACTTGCTT ATTATATTTG
     TTTTAAGGAA AGCTGACAGT CTGTTATCCT AACGACACCA GATGAACGAA TAATATAAAC

541  TAGAGTCTAG AATGCAATCT CACTACACTA TAGACATCTC ANNCTAACGT AGGACAATTC
     ATCTCAGATC TTACGTTAGA GTGATGTGAT ATCTGTAGAG TNNGATTGCA TCCTGTTAAG

601  TGAGAAACTA TTCCAGACCT CCTTATGGGC TTAGCCAAGG NTATCCTTCA GCTGGCATTG
     ACTCTTTGAT AAGGTCTGGA GGAATACCCG AATCGGTTCC NATAGGAAGT CGACCGTAAC

661  CAGGGTGACT TCTNCCTCNN AATCCAGCTC TCTNTCACAG ATGTGATCCA AGAGACACTC
     GTCCCACTGA AGANGGAGNN TTAGGTCGAG AGANAGTGTC TACACTAGGT TCTCTGTGAG

721  ACAATTAATC AACTAGCATT CTAAATTTCA ATTCCAGATC TATTACCTTA ATATGGTAGC
     TGTTAATTAG TTGATCGTAA GATTTAAAGT TAAGGTCTAG ATAATGGAAT TATACCATCG
```

FIGURE 43B

```
781 TGAAGCTTTN NTCACTGTCA ATTCTGATCA GATATATGAC AATTTTAAAT TATTTGCAGT
    ACTTCGAAAN NAGTGACAGT TAAGACTAGT CTATATACTG TTAAAATTTA ATAAACGTCA

841 GTGTAAGAAA CGCTTCAGGT AGTTTAAATT TAAGGCT
    CACATTCTTT GCGAAGTCCA TCAAATTTAA ATTCCGA
```

FIGURE 44A

```
            10         20         30         40         50         60
             |          |          |          |          |          |
  1 CTCCTTTGGC CCCTGCCAGC TGGGCATTTT TAACCTAGTT TACACAGTGT CTTTTTTTCC
    GAGGAAACCG GGGACGGTCG ACCCGTAAAA ATTGGATCAA ATGTGTCACA GAAAAAAAGG

 61 TTATTTTAAA TTGGTTGTTC CAGATTCGGT AATATCAATT TTTAATATTA CACTTAAATG
    AATAAAATTT AACCAACAAG GTCTAAGCCA TTATAGTTAA AAATTATAAT GTGAATTTAC

121 AGTACCAGAA CTTTATCTTC AACCTTTTTC TCATTAGGCC TACAACATAG GACATCTCGG
    TCATGGTCTT GAAATAGAAG TTGGAAAAAG AGTAATCCGG ATGTTGTATC CTGTAGAGCC

181 ATAGAATTTC CTTTTCTTTT TGCTACTATA AGCTGCTAAA ATCCTCAGAA CATCAGATTT
    TATCTTAAAG GAAAAGAAAA ACGATGATAT TCGACGATTT TAGGAGTCTT GTAGTCTAAA

241 AGAAATGTTC TTATTAGTGG TAGTGAGCAT TTGCTATTTC CTACCACTAG CTTACAAATA
    TCTTTACAAG AATAATCACC ATCACTCGTA AACGATAAAG GATGGTGATC GAATGTTTAT

301 TAATAAGCAA GTAGACCCCA CAGGCCAAAT TCCTATTTGT TCTACAGTCG AAAGGGAATT
    ATTATTCGTT CATCTGGGGT GTCCGGTTTA AGGATAAACA AGATGTCAGC TTTCCCTTAA

361 TTTTAAAATT TAATTTCCAC TAAAGAGAAA AATATATTAA CAATCAAATT GACAGTCGAT
    AAAATTTTAA ATTAAAGGTG ATTTCTCTTT TTATATAATT GTTAGTTTAA CTGTCAGCTA

421 TTTAATTGCT ATGTGTAATT GTTTTCCCTC ATTATTTATA ACAATTCATA CTACAATTTA
    AAATTAACGA TACACATTAA CAAAAGGGAG TAATAAATAT TGTTAAGTAT GATGTTAAAT

481 ATTTAGTAAA CATTTTTGTA GACCATATTT AAAACAAAGA TACTGAAAGT TAATATAAAC
    TAAATCATTT GTAAAAACAT CTGGTATAAA TTTTGTTTCT ATGACTTTCA ATTATATTTG

541 CCAGTGCATG CTCTCTGTAG GCCACAGCCA TAACCTGTAA GCACAGAAAA ATTTGTTCTG
    GGTCACGTAC GAGAGACATC CGGTGTCGGT ATTGGACATT CGTGTCTTTT TAAACAAGAC

601 TTACTCTAAA CATCTATACT GGCCAAATTC CAATGCTCGA ATTTAACCCC GGGATATAAC
    AATGAGATTT GTAGATATGA CCGGTTTAAG GTTACGAGCT TAAATTGGGG CCCTATATTG

661 CTAGTAAATG TGTCCTCTCT GTCAAGGTGG GCATGTCACA GAATACAGAA CAATCAATGG
    GATCATTTAC ACAGGAGAGA CAGTTCCACC CGTACAGTGT CTTATGTCTT GTTAGTTACC

721 TATTCATAAA GTTTTAAGAA AATGATTCTA CACATGTAAA ACCCACTATA ACTTTTTACA
    ATAAGTATTT CAAAATTCTT TTACTAAGAT GTGTACATTT TGGGTGATAT TGAAAAATGT
```

FIGURE 44B

```
781 TTGGGGGAGA GAAAAAAAGA GATAATTTTT ACCTTACCTT ATTTCCTCTG AAAACTTTCC
    AACCCCCTCT CTTTTTTTCT CTATTAAAAA TGGAATGGAA TAAAGGAGAC TTTTGAAAGG

841 CATATCTGGC AATTACAATT TTCCCAGAGC AATTGATTTT CATGTCCCGT TCC
    GTATAGACCG TTAATGTTAA AAGGGTCTCG TTAACTAAAA GTACAGGGCA AGG
```

FIGURE 45A

```
              10         20         30         40         50         60
               |          |          |          |          |          |
  1 GATGCTATTT GGGCAATTTC TTATTGACAG TTTTGAAATG TTAGGCTTTT ATCTCCATTT
    CTACGATAAA CCCGTTAAAG AATAACTGTC AAAACTTTAC AATCCGAAAA TAGAGGTAAA

 61 TTTAGTACTT AAATTTTCCA ACATGGGTGT TGCTTGTTAT TTTATCAGTA TAAAATAGAA
    AAATCATGAA TTTAAAAGGT TGTACCCACA ACGAACAATA AAATAGTCAT ATTTTATCTT

121 GAGTGGTTCT GTTCTGGAAT TTAGTATATA CATGAGTATC TAGTGTATGT CAGCCATGAA
    CTCACCAAGA CAAGACCTTA AATCATATAT GTACTCATAG ATCACATACA GTCGGTACTT

181 AATGAACCTT TCAGATGTTT AACTTCAGGG AACCTAATTG AGTCATTGCT CCAGACATTG
    TTACTTGGAA AGTCTACAAA TTGAAGTCCC TTGGATTAAC TCAGTAACGA GGTCTGTAAC

241 TTGCTTTGAA CCCACTATAT TNNNNNNNCT CGGGCAATGA CTCAGTGTGG CAAGGATACT
    AACGAAACTT GGGTGATATA ANNNNNNNGA GCCCGTTACT GAGTCACACC GTTCCTATGA

301 ACTGCAGGCC TGTTTCTGGA AGGCACTGGA CTCCTCTGAT GCAAACTTTG GCCAGGGACT
    TGACGTCCGG ACAAAGACCT TCCGTGACCT GAGGAGACTA CGTTTGAAAC CGGTCCCTGA

361 CCTTGATAGC TCTTAAATAG ATGCTGCACC AACACTCTCT TTCTTTTCTC TCTTTTTCTT
    GGAACTATCG AGAATTTATC TACGACGTGG TTGTGAGAGA AAGAAAAGAG AGAAAAAGAA

421 TATTCAATAT TAGACTACAA GCAGTCTAAG GACTTCTCAG GGTTTCTAGC TCTCTCTCAT
    ATAAGTTATA ATCTGATGTT CGTCAGATTC CTGAAGAGTC CCAAAGATCG AGAGAGAGTA

481 TTCACACATG CTTTCCTAGT AATCTCTACT CATATATCTT ACTGCTACGC TGGGGCCAGA
    AAGTGTGTAC GAAAGGATCA TTAGAGATGA GTATATAGAA TGACGATGCG ACCCCGGTCT

541 TAACNNNNNN CTTCCATTTT GTTTTTATCT CTATTCTTCT TCCCCTTCTG CTTTCATTAT
    ATTGNNNNNN GAAGGTAAAA CAAAAATAGA GATAAGAAGA AGGGGAAGAC GAAAGTAATA

601 TGAAACTTTC TGCTTTCATT ATTGAAACTT TCCCAGATTT GTTCTGCTTA ACCTGGCATT
    ACTTTGAAAG ACGAAAGTAA TAACTTTGAA AGGGTCTAAA CAAGACGAAT TGGACCGTAA

661 GGAACTGTTT CCTCTTCCCT GTGCTGCTTT CTCCCATTGC CATGTCCTTT TTTTTTTTTT
    CCTTGACAAA GGAGAAGGGA CACGACGAAA GAGGGTAACG GTACAGGAAA AAAAAAAAAA

721 TTTTTTTTTT TGAGACAGTG TCACTCTGTT GCCCAGGCTG GAGTGCAATG GTGCAATCTT
    AAAAAAAAAA ACTCTGTCAC AGTGAGACAA CGGGTCCGAC CTCACGTTAC CACGTTAGAA
```

FIGURE 45B

```
781 GGCCACTGCA ACCCCGACTC CGGGTTCAAG TGATTCTCTA CCTGCCTCAG CCTCCTGAGT
    CCGGTGACGT TGGGGCTGAG GCCCAAGTTC ACTAAGAGAT GGACGGAGTC GGAGGACTCA

841 AGCTGGGATT ACAGGTGCCA CCACTATGCC GGCTGATTTT GTATTTTAGT AGAGATGGGT
    TCGACCCTAA TGTCCACGGT GGTGATACGG CCGACTAAAA CATAAAATCA TCTCTACCCA

901 TCACATGCAG ATCAGCTGTT CCGACTCTGA CCAGNNNNNN NNNNNNNNNN ATCAAAGTCA
    AGTGTACGTC TAGTCGACAA GGCTGAGACT GGTCNNNNNN NNNNNNNNNN TAGTTTCAGT

961 GCCAAAGTGC TAGGCTTAGA GTAATTGTGT AATTTCCACA CAAGTGCAAC CTAGTGTAAT
    CGGTTTCACG ATCCGAATCT CATTAACACA TTAAAGGTGT GTTCACGTTG GATCACATTA

1021 GCCTCAAGAA TGTNNNTATG AATGTCTCGA ACGTTAGTAA CTAATAACAA GTAGTTAGTT
     CGGAGTTCTT ACANNNATAC TTACAGAGCT TGCAATCATT GATTATTGTT CATCAATCAA

1081 TATAGATGTA TCCTAGTATG TAGCA
     ATATCTACAT AGGATCATAC ATCGT
```

FIGURE 46A

```
         10         20         30         40         50         60
          |          |          |          |          |          |
  1 CACAAAAAAA GATTATTAGC CACAAAAAAA CCTTGAAGTA ACGCATTAAA ATGTTAATGG
    GTGTTTTTTT CTAATAATCG GTGTTTTTTT GGAACTTCAT TGCGTAATTT TACAATTACC

 61 ATTCACTTTA TTGAGCATCT GCTCATAATA CTTTAATGAG TGCAAAGTGC TTTGAATATA
    TAAGTGAAAT AACTCGTAGA CGAGTATTAT GAAATTACTC ACGTTTCACG AAACTTATAT

121 ATACGTCATT TAAACCTTAC CATAATTCTG AGGAATTGCT ACCTCCACTT CACAGATGGG
    TATGCAGTAA ATTTGGAATG GTATTAAGAC TCCTTAACGA TGGAGGTGAA GTGTCTACCC

181 GCACAGGAGG CTTAGATAAC ATGCCCAAAG TCATGCTTCT AGTAAATGGA TATAATTAAG
    CGTGTCCTCC GAATCTATTG TACGGGTTTC AGTACGAAGA TCATTTACCT ATATTAATTC

241 ATTCAAATTA TTGATAAGAA TTTGATCTGC CTTACCAGTA TCTAGTAGTA AATCTAAAAG
    TAAGTTTAAT AACTATTCTT AAACTAGACG GAATGGTCAT AGATCATCAT TTAGATTTTC

301 CGCTTTCCAG AGCATGTGCT GTTGATAGAG CTTGATGTCT AACTCTCTGA AATTTTCCAT
    GCGAAAGGTC TCGTACACGA CAACTATCTC GAACTACAGA TTGAGAGACT TTAAAAGGTA

361 TCTTATTTGT CTCACTGGTA TATAGTTATT TTTTACTACT TTCATACACC TACTAAGAAG
    AGAATAAACA GAGTGACCAT ATATCAATAA AAAATGATGA AAGTATGTGG ATGATTCTTC

421 ACAGGAGGAT CAAAGATAGG ATTTCATTTA GAATGCCTAA AGCTTCACGT ATTTTAATTC
    TGTCCTCCTA GTTTCTATCC TAAAGTAAAT CTTACGGATT TCGAAGTGCA TAAAATTAAG

481 AGAATAAGAT TCAGGCAGAC CACCAGTATA TGCCATGGTC CCTGGTTATC TTTCAGCAGG
    TCTTATTCTA AGTCCGTCTG GTGGTCATAT ACGGTACCAG GGACCAATAG AAAGTCGTCC

541 TGACCGAGAA AGAAAACATG GTAATGTTTA TGAAATGGTG GGTTCTTGTA GTTTCACTTC
    ACTGGCTCTT TCTTTTGTAC CATTACAAAT ACTTTACCAC CCAAGAACAT CAAAGTGAAG

601 AACATATCTG CCTTTACTGT ATTAAGATGA TGGATTAACT TATTCTTGAT ATGGGCATGT
    TTGTATAGAC GGAAATGACA TAATTCTACT ACCTAATTGA ATAAGAACTA TACCCGTACA

661 AAAACAATAT ACTTTTACTA AACAGCTACA GAGAGACAAA TGTGTTTCCA GACAAACTTA
    TTTTGTTATA TGAAAATGAT TTGTCGATGT CTCTCTGTTT ACACAAAGGT CTGTTTGAAT

721 AGAGACTGAG TGTTCAAACT GAATAATCTC GACCTTAATT GTAACTATAT TTTATGAAAT
    TCTCTGACTC ACAAGTTTGA CTTATTAGAG CTGGAATTAA CATTGATATA AAATACTTTA
```

FIGURE 46B

```
781 CCAGCTGTAA GGCAAAACAG ACTCTTGGCT ACACGGCATT TGTCTGTTAA TGATACTCAA
    GGTCGACATT CCGTTTTGTC TGAGAACCGA TGTGCCGTAA ACAGACAATT ACTATGAGTT

841 CCTTAACCGT CACTTAATAA TGCTGAATAA TGTCATTAAT CTGAGATGTT AGTATGATCA
    GGAATTGGCA GTGAATTATT ACGACTTATT ACAGTAATTA GACTCTACAA TCATACTAGT

901 ATGGGAATCA CTGCTGAGCT CTCGAAGCCC
    TACCCTTAGT GACGACTCGA GAGCTTCGGG
```

FIGURE 47A

```
                                                       261 CTCAAAAGGGGCCGGATTTCCTT  -239
CTCCTGGAGGCAGATGTTGCCCTCTCTCTCTCGCTCGGATTGGTTCAGTGCACTCTAGAAACACTGCTGTGGTGGAGAAACTGGACCCCAGGTCTGGAGCGAATTCCAGCCTGCAGGGCT  -120
GATAAGCGAGGCATTAGTGAGATTGAGAGAGACTTTACCCCCGCCGTGGTGGTTGGAGGGCGCGCAGTAGAGCAGGCAGCACAGGCGCCGGGTCCCGGGAGGCCGGCTCTGCTCGGCGCCGAG    -1

ATG TGG AAT CTC CTT CAC GAA ACC GAC TCG GCT GTG GCC ACC GCG CGC CGC CCG CGC TGG CTG TGC GCT GGG GCG CTG GTG CTG GCG GGT   90
Met Trp Asn Leu Leu His Glu Thr Asp Ser Ala Val Ala Thr Ala Arg Arg Pro Arg Trp Leu Cys Ala Gly Ala Leu Val Leu Ala Gly   30
                                        Intron
GGC TTC TTT CTC CTC GGC TTC CTC TTC GGG TGG|TTT ATA AAA TCC TCC AAT GAA GCT ACT AAC ATT ACT CCA AAG CAT AAT ATG AAA GCA  180
Gly Phe Phe Leu Leu Gly Phe Leu Phe Gly Trp|Phe Ile Lys Ser Ser Asn Glu Ala Thr Asn Ile Thr Pro Lys His Asn Met Lys Ala   60

TTT TTG GAT GAA TTG AAA GCT GAG AAC ATC AAG AAG TTC TTA TAT AAT TTT ACA CAG ATA CCA CAT TTA GCA GGA ACA GAA CAA AAC TTT  270
Phe Leu Asp Glu Leu Lys Ala Glu Asn Ile Lys Lys Phe Leu Tyr Asn Phe Thr Gln Ile Pro His Leu Ala Gly Thr Glu Gln Asn Phe   90
                                                              *
CAG CTT GCA AAG CAA ATT CAA TCC CAG TGG AAA GAA TTT GGC CTG GAT TCT GTT GAG CTA GCA CAT TAT GAT GTC CTG TTG TCC TAC CCA  360
Gln Leu Ala Lys Gln Ile Gln Ser Gln Trp Lys Glu Phe Gly Leu Asp Ser Val Glu Leu Ala His Tyr Asp Val Leu Leu Ser Tyr Pro  120
                                                                    Intron
AAT AAG ACT CAT CCC AAC TAC ATC TCA ATA ATT AAT GAA GAT GGA AAT GAG|ATT TTC AAC ACA TCA TTA TTT GAA CCA CCT CCT CCA GGA  450
Asn Lys Thr His Pro Asn Tyr Ile Ser Ile Ile Asn Glu Asp Gly Asn Glu|Ile Phe Asn Thr Ser Leu Phe Glu Pro Pro Pro Pro Gly  150
 *                                                                          *
TAT GAA AAT GTT TCG GAT ATT GTA CCA CCT TTC AGT GCT TTC TCT CCT CAA GGA ATG CCA GAG GGC GAT CTA GTG TAT GTT AAC TAT GCA  540
Tyr Glu Asn Val Ser Asp Ile Val Pro Pro Phe Ser Ala Phe Ser Pro Gln Gly Met Pro Glu Gly Asp Leu Val Tyr Val Asn Tyr Ala  180
         *
```

FIGURE 47B

CGA ACT GAA GAC TTC TTT AAA TTG GAA CGG GAC ATG AAA ATC AAT TGC TCT GGG AAA ATT GTA ATT GCC AGA TAT GGG AAA GTT TTC AGA 630

Arg Thr Glu Asp Phe Phe Lys Leu Glu Arg Asp Met Lys Ile Asn Cys Ser Gly Lys Ile Val Ile Ala Arg Tyr Gly Lys Val Phe Arg 210

Intron

GGA AAT AAG GTT AAA AAT GCC CAG CTG GCA GGG GCC AAA GGA GTC ATT CTC TAC TCC GAC CCT GCT GAC TAC TTT GCT CCT GGG GTG AAG 720

Gly Asn Lys Val Lys Asn Ala Gln Leu Ala Gly Ala Lys Gly Val Ile Leu Tyr Ser Asp Pro Ala Asp Tyr Phe Ala Pro Gly Val Lys 240

TCC TAT CCA GAT GGT TGG AAT CTT CCT GGA GGT GGT GTC CAG CGT GGA AAT ATC CTA AAT CTG AAT GGT GCA GGA GAC CCT CTC ACA CCA 810

Ser Tyr Pro Asp Gly Trp Asn Leu Pro Gly Gly Gly Val Gln Arg Gly Asn Ile Leu Asn Leu Asn Gly Ala Gly Asp Pro Leu Thr Pro 270

GGT TAC CCA GCA AAT GAA TAT GCT TAT AGG CGT GGA ATT GCA GAG GCT GTT GGT CTT CCA AGT ATT CCT GTT CAT CCA ATT GGA TAC TAT 900

Gly Tyr Pro Ala Asn Glu Tyr Ala Tyr Arg Arg Gly Ile Ala Glu Ala Val Gly Leu Pro Ser Ile Pro Val His Pro Ile Gly Tyr Tyr 300

Intron

GAT GCA CAG AAG CTC CTA GAA AAA ATG GGT GGC TCA GCA CCA CCA GAT AGC AGC TGG AGA GGA AGT CTC AAA GTG CCC TAC AAT GTT GGA 990

Asp Ala Gln Lys Leu Leu Glu Lys Met Gly Gly Ser Ala Pro Pro Asp Ser Ser Trp Arg Gly Ser Leu Lys Val Pro Tyr Asn Val Gly 330

CCT GGC TTT ACT GGA AAC TTT TCT ACA CAA AAA GTC AAG ATG CAC ATC CAC TCT ACC AAT GAA GTG ACA AGA ATT TAC AAT GTG ATA GGT 1080

Pro Gly Phe Thr Gly Asn Phe Ser Thr Gln Lys Val Lys Met His Ile His Ser Thr Asn Glu Val Thr Arg Ile Tyr Asn Val Ile Gly 360

FIGURE 47C

ACT CTC AGA GGA GCA GTG GAA CCA GAC AGA TAT GTC ATT CTG GGA GGT CAC CGG GAC TCA TGG GTG TTT GGT GGT ATT GAC CCT CAG AGT 1170
Thr Leu Arg Gly Ala Val Glu Pro Asp Arg Tyr Val Ile Leu Gly Gly His Arg Asp Ser Trp Val Phe Gly Gly Ile Asp Pro Gln Ser 390

GGA GCA GCT GTT GTT CAT GAA ATT GTG AGG AGC TTT GGA ACA CTG AAA AAG GAA GGG TGG AGA CCT AGA AGA ACA ATT TTG TTT GCA AGC 1260
Gly Ala Ala Val Val His Glu Ile Val Arg Ser Phe Gly Thr Leu Lys Lys Glu Gly Trp Arg Pro Arg Arg Thr Ile Leu Phe Ala Ser 420

TGG GAT GCA GAA GAA TTT GGT CTT CTT GGT TCT ACT GAG TGG GCA GAG GAG AAT TCA AGA CTC CTT CAA GAG CGT GGC GTG GCT TAT ATT 1350
Trp Asp Ala Glu Glu Phe Gly Leu Leu Gly Ser Thr Glu Trp Ala Glu Glu Asn Ser Arg Leu Leu Gln Glu Arg Gly Val Ala Tyr Ile 450

AAT GCT GAC TCA TCT ATA GAA GGA AAC TAC ACT CTG AGA GTT GAT TGT ACA CCG CTG ATG TAC AGC TTG GTA CAC AAC CTA ACA AAA GAG 1440
Asn Ala Asp Ser Ser Ile Glu Gly Asn Tyr Thr Leu Arg Val Asp Cys Thr Pro Leu Met Tyr Ser Leu Val His Asn Leu Thr Lys Glu 480

CTG AAA AGC CCT GAT GAA GGC TTT GAA GGC AAA TCT CTT TAT GAA AGT TGG ACT AAA AAA AGT CCT TCC CCA GAG TTC AGT GGC ATG CCC 1530
Leu Lys Ser Pro Asp Glu Gly Phe Glu Gly Lys Ser Leu Tyr Glu Ser Trp Thr Lys Lys Ser Pro Ser Pro Glu Phe Ser Gly Met Pro 510

AGG ATA AGC AAA TTG GGA TCT GGA AAT GAT TTT GAG GTG TTC TTC CAA CGA CTT GGA ATT GCT TCA GGC AGA GCA CGG TAT ACT AAA AAT 1620
Arg Ile Ser Lys Leu Gly Ser Gly Asn Asp Phe Glu Val Phe Phe Gln Arg Leu Gly Ile Ala Ser Gly Arg Ala Arg Tyr Thr Lys Asn 540

TGG GAA ACA AAC AAA TTC AGC GGC TAT CCA CTG TAT CAC AGT GTC TAT GAA ACA TAT GAG TTG GTG GAA AAG TTT TAT GAT CCA ATG TTT 1710
Trp Glu Thr Asn Lys Phe Ser Gly Tyr Pro Leu Tyr His Ser Val Tyr Glu Thr Tyr Glu Leu Val Glu Lys Phe Tyr Asp Pro Met Phe 570

FIGURE 47D

AAA TAT CAC CTC ACT GTG GCC CAG GTT CGA GGA GGG ATG GTG TTT GAG CTA GCC AAT TCC ATA GTG CTC CCT TTT GAT TGT CGA GAT TAT 1800
Lys Tyr His Leu Thr Val Ala Gln Val Arg Gly Gly Met Val Phe Glu Leu Ala Asn Ser Ile Val Leu Pro Phe Asp Cys Arg Asp Tyr 600

GCT GTA GTT TTA AGA AAG TAT GCT GAC AAA ATC TAC AGT ATT TCT ATG AAA CAT CCA CAG GAA ATG AAG ACA TAC AGT GTA TCA TTT GAT 1890
Ala Val Val Leu Arg Lys Tyr Ala Asp Lys Ile Tyr Ser Ile Ser Met Lys His Pro Gln Glu Met Lys Thr Tyr Ser Val Ser Phe Asp 630

TCA CTT TTT TCT GCA GTA AAG AAT TTT ACA GAA ATT GCT TCC AAG TTC AGT GAG AGA CTC CAG GAC TTT GAC AAA AGC AAC CCA ATA GTA 1980
Ser Leu Phe Ser Ala Val Lys Asn* Phe Thr Glu Ile Ala Ser Lys Phe Ser Glu Arg Leu Gln Asp Phe Asp Lys Ser Asn Pro Ile Val 660

TTA AGA ATG ATG AAT GAT CAA CTC ATG TTT CTG GAA AGA GCA TTT ATT GAT CCA TTA GGG TTA CCA GAC AGG CCT TTT TAT AGG CAT GTC 2070
Leu Arg Met Met Asn Asp Gln Leu Met Phe Leu Glu Arg Ala Phe Ile Asp Pro Leu Gly Leu Pro Asp Arg Pro Phe Tyr Arg His Val 690

ATC TAT GCT CCA AGC AGC CAC AAC AAG TAT GCA GGG GAG TCA TTC CCA GGA ATT TAT GAT GCT CTG TTT GAT ATT GAA AGC AAA GTG GAC 2160
Ile Tyr Ala Pro Ser Ser His Asn Lys Tyr Ala Gly Glu Ser Phe Pro Gly Ile Tyr Asp Ala Leu Phe Asp Ile Glu Ser Lys Val Asp 720

CCT TCC AAG GCC TGG GGA GAA GTG AAG AGA CAG ATT TAT GTT GCA GCC TTC ACA GTG CAG GCA GCT GCA GAG ACT TTG AGT GAA GTA GCC 2250
Pro Ser Lys Ala Trp Gly Glu Val Lys Arg Gln Ile Tyr Val Ala Ala Phe Thr Val Gln Ala Ala Ala Glu Thr Leu Ser Glu Val Ala 750

TAA GAGGATTCTTTAGAGAATCCGTATTGAATTTGTGTGGTATGTCACTCAGAAAGAATCGTAATGGGTATATTGATAAATTTTAAAATTGGTATATTTGAAATAAAGTTGAATATTA 2368
\-\-\-

TATATAAAAAAAAAAAAAAAAAAAA 2393

FIGURE 48

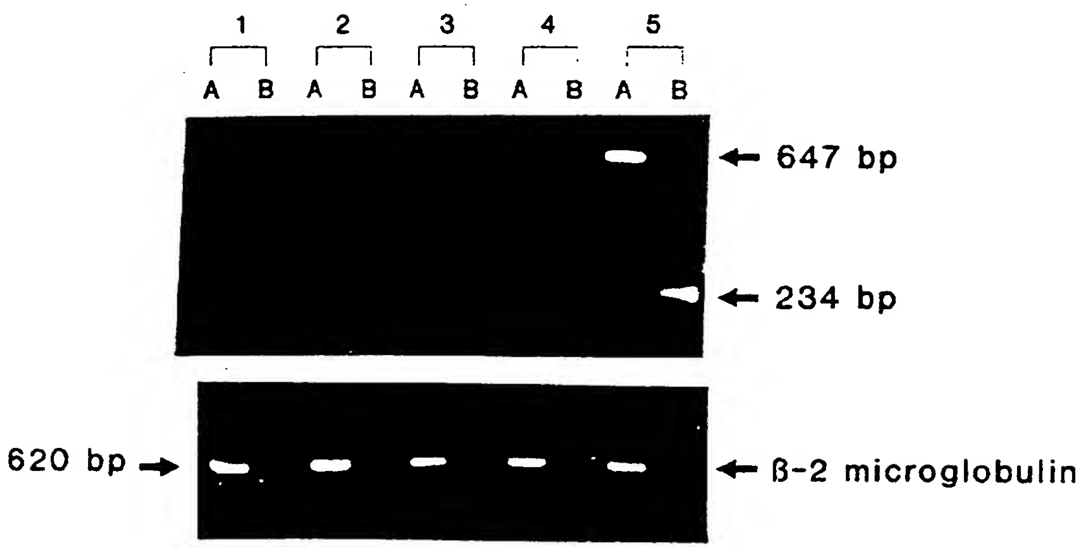


FIGURE 49

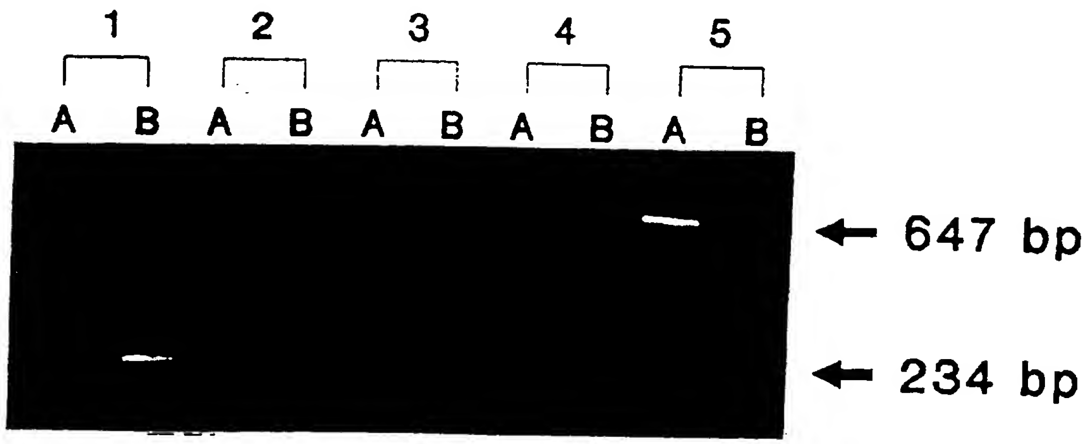


FIGURE 50

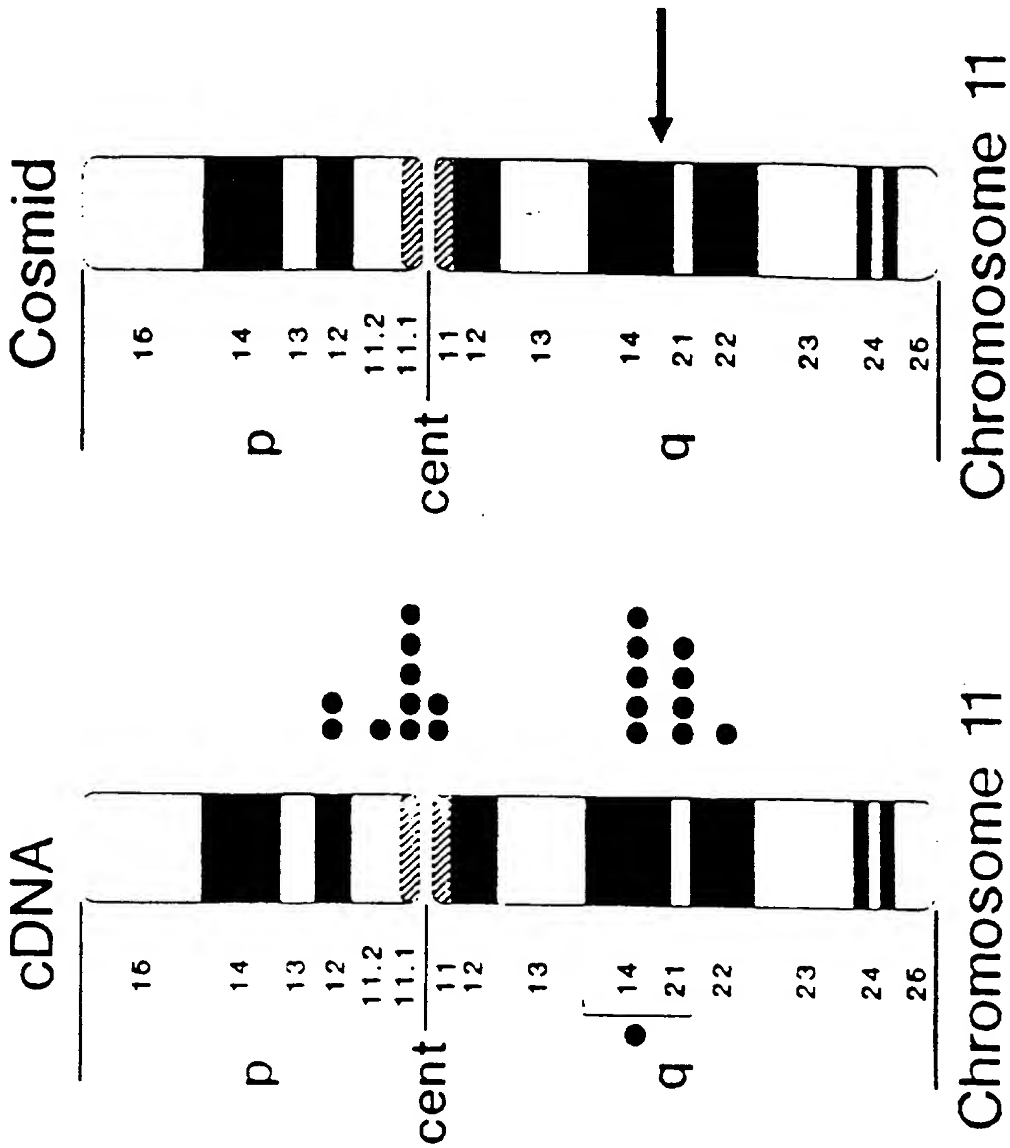

cDNA
p
cent
q
16
14
13
12
11.2
11.1
11
12
13
14
21
22
23
24
26
Chromosome 11
Cosmid
p
cent
q
16
14
13
12
11.2
11.1
11
12
13
14
21
22
23
24
26
Chromosome 11

FIGURE 51

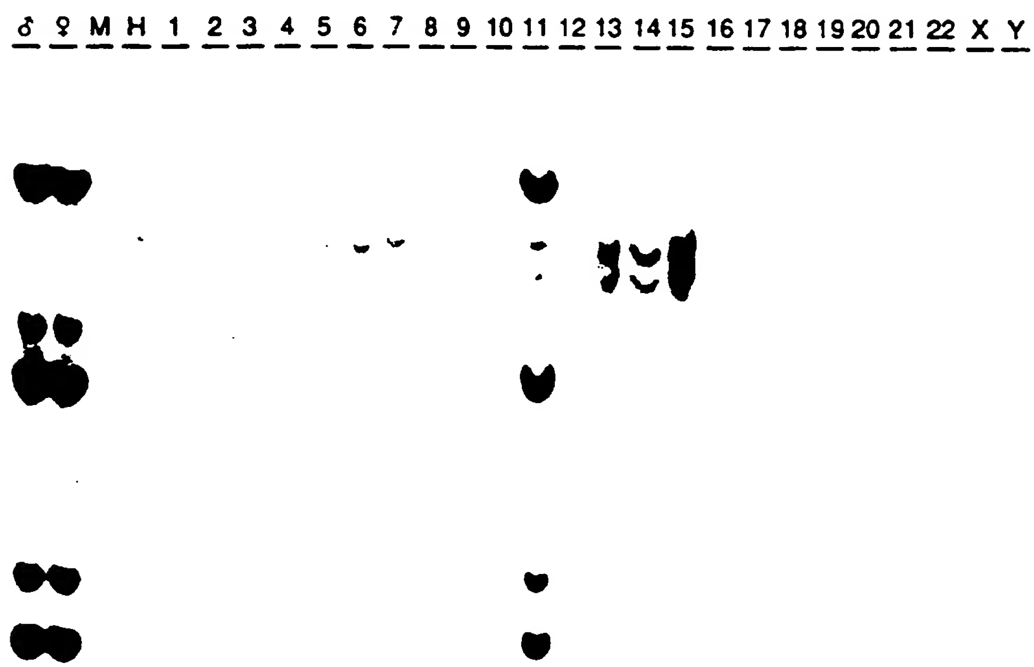


FIGURE 52

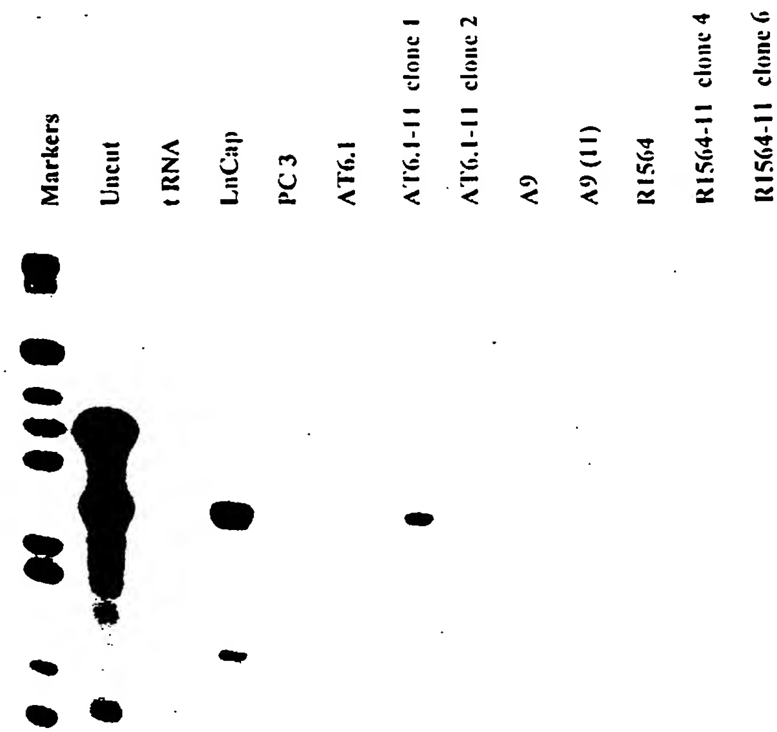

Markers
Uncut
t RNA
LnCap
PC 3
AT6.1
AT6.1-11 clone 1
AT6.1-11 clone 2
A9
A9 (11)
R1564
R1564-11 clone 4
R1564-11 clone 6

FIGURE 53

TISSUE/ CELL LINE	CANCER CELL TYPE	[1]PSM DNA	[2]PSM RNA
HUMAN PROSTATE	N.A.	+	+
HUMAN MAMMARY	N.A.	+	-
AT6.1	RAT PROSTATIC ADENOCARCINOMA	-	-
AT6.1-11-CL1	"	+	+
AT6.1-11-CL2	"	-	-
R1564	RAT MAMMARY ADENOCARCINOMA	-	-
R1564-11-CL2	"	+	-
R1564-11-CL4	"	+	-
R1564-11-CL5	"	+	-
R1564-11-CL6	"	+	-
A9	MOUSE FIBROSARCOMA	-	-
A9(11)	"	+	-

FIGURE 54

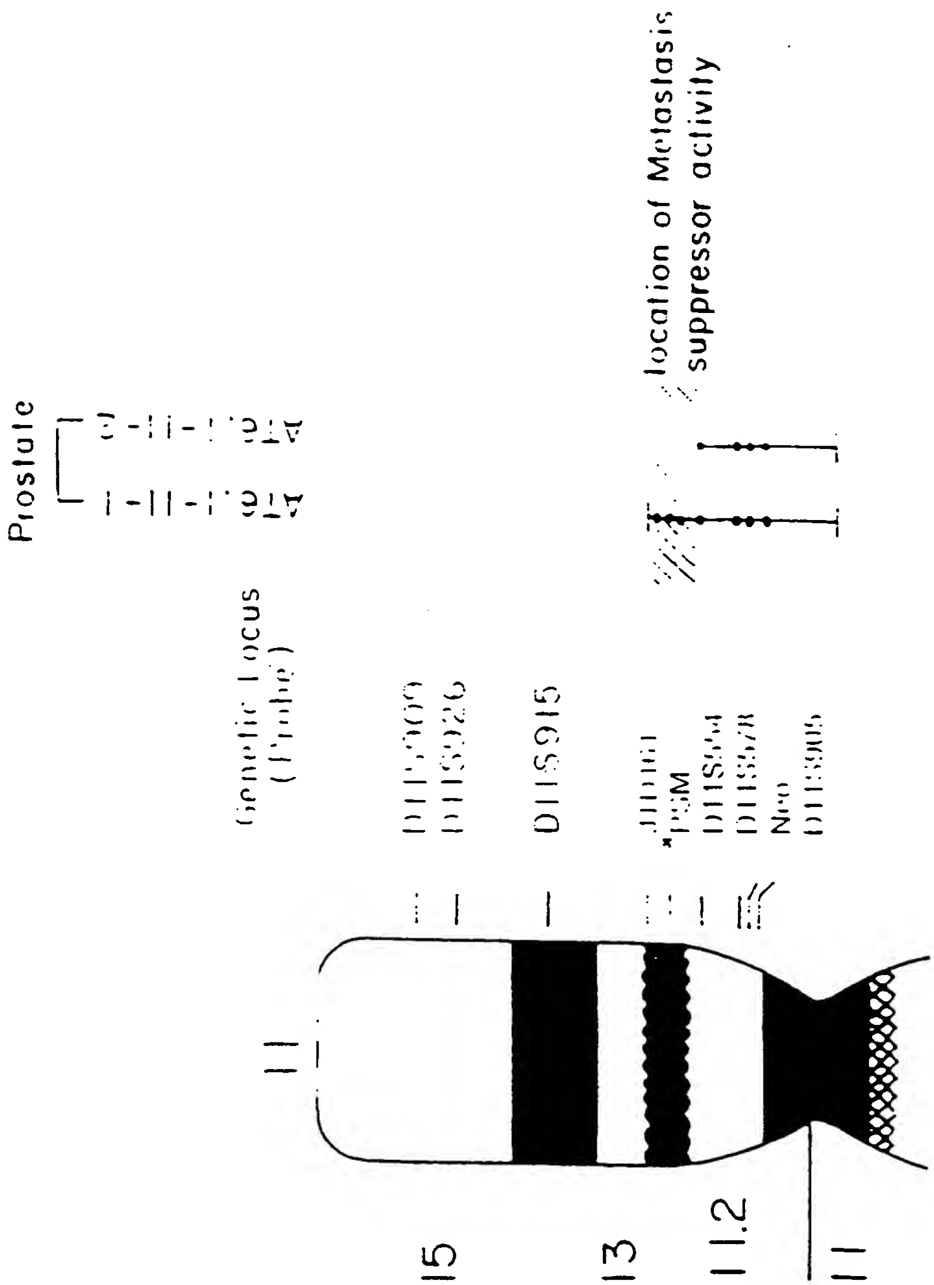

Prostate
Genetic Locus
D11S915
PSM
Neo
location of Metastasis
suppressor activity
11
15
13
11.2
11

FIGURE 55

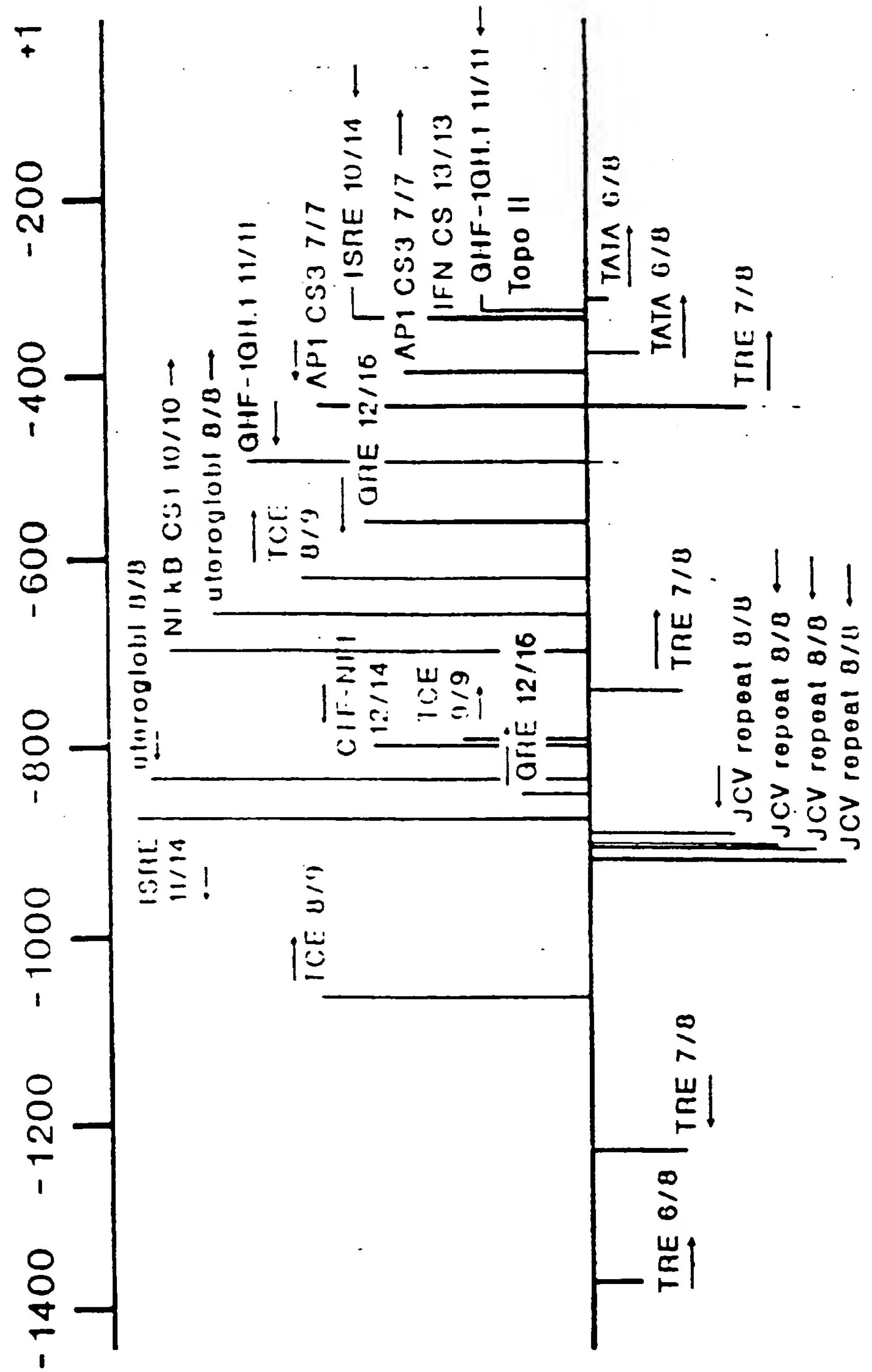

+1
-200
-400
-600
-800
-1000
-1200
-1400
ISRE 10/14
AP1 CS3 7/7
IFN CS 13/13
Topo II
TATA 6/8
TRE 7/8
GRE 12/16
JCV repeat 8/8
TRE 6/8

FIGURE 56

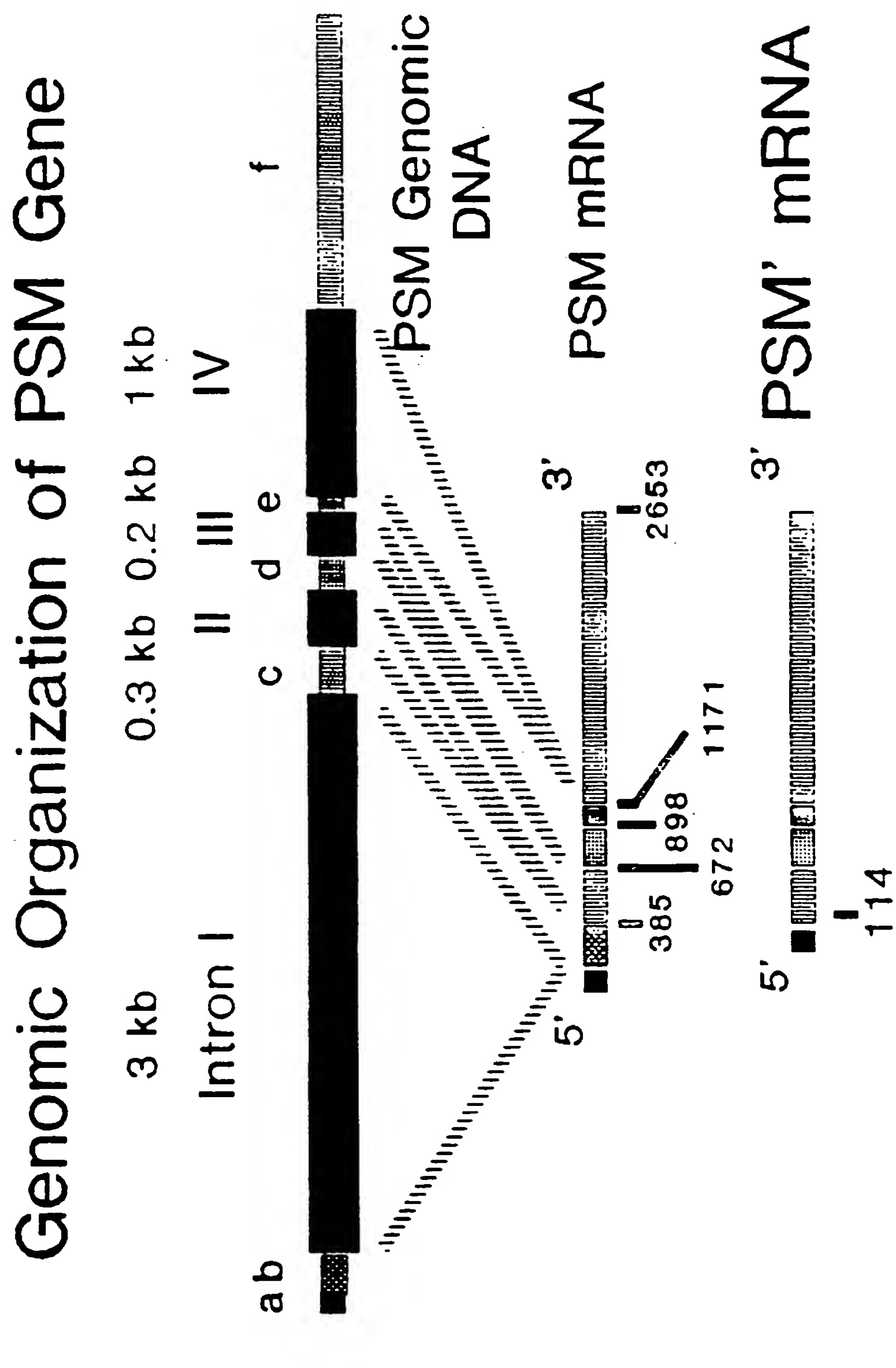

Genomic Organization of PSM Gene
3 kb
0.3 kb
0.2 kb
1 kb
Intron I
II
III
IV
a b
c
d
e
f
PSM Genomic DNA
5'
3'
385
672
898
1171
2653
PSM mRNA
5'
3'
114
PSM' mRNA

FIGURE 57

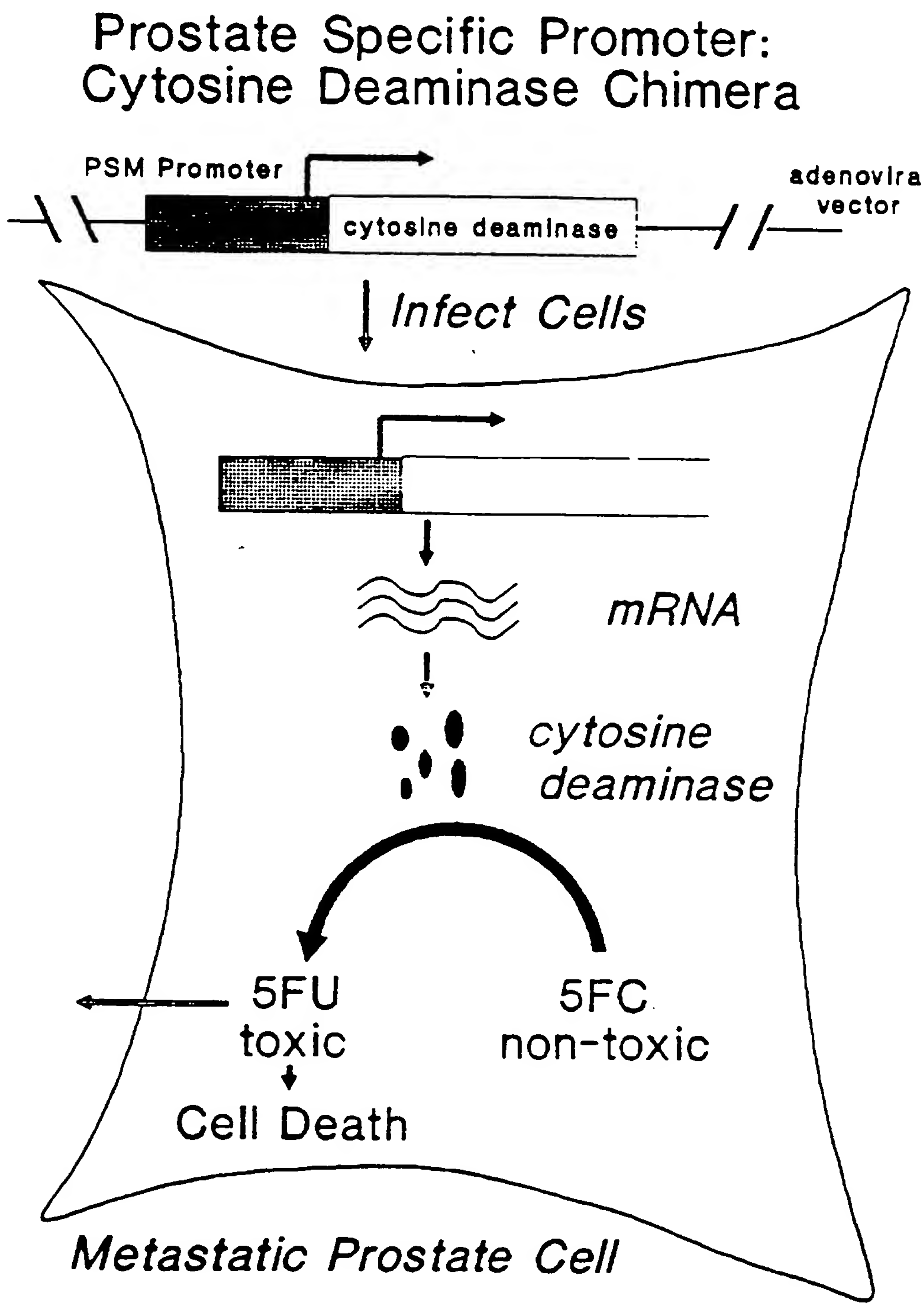

Prostate Specific Promoter:
Cytosine Deaminase Chimera
PSM Promoter
cytosine deaminase
adenoviral vector
Infect Cells
mRNA
cytosine deaminase
5FU
toxic
Cell Death
5FC
non-toxic
Metastatic Prostate Cell

FIGURE 58A

```
               10         20         30         40         50         60
                |          |          |          |          |          |
  1 GCGCCTTAAA AAAAAAAAAC TTTCTTGGAA AATGTCCAGC TCTTGCTTAA ATATAAAAAT
    CGCGGAATTT TTTTTTTTTG AAAGAACCTT TTACAGGTCG AGAACGAATT TATATTTTTA

 61 GAAAGGAAGA AAGAGACTCT CCTCTCTCCA CTCCTATAAT TATGAGGAAC TTTTATTCAA
    CTTTCCTTCT TTCTCTGAGA GGAGAGAGGT GAGGATATTA ATACTCCTTG AAAATAAGTT

121 CTCTGAAATT CTATACAATC TCTACAATAC TCTACTGAAT AAAAGCAGAG CAGAAAAAGC
    GAGACTTTAA GATATGTTAG AGATGTTATG AGATGACTTA TTTTCGTCTC GTCTTTTTCG

181 TGCGCTTTTT TTCCATAGTC GGGAATGCTT GTCATCAGTG TAAATCACCA CCGCGCCCTT
    ACGCGAAAAA AAGGTATCAG CCCTTACGAA CAGTAGTCAC ATTTAGTGGT GGCGCGGGAA

241 TTTCCTAAAG AATATTATTG TTATTAATAA ACATGTAGGG TATTATCCTC CACTTACATT
    AAAGGATTTC TTATAATAAC AATAATTATT TGTACATCCC ATAATAGGAG GTGAATGTAA

301 ACAAAACCAT TTTTTAAAGC CGGGCGTGGT GGCTCACGCC TGTAATCCCA GCACTTTGGG
    TGTTTTGGTA AAAAATTTCG GCCCGCACCA CCGAGTGCGG ACATTAGGGT CGTGAAACCC

361 AGGCCCAGAC AGGCGGATCA CGAAGTCGAG AAATCGAGAC CATCCTGGCC AACATGGTGA
    TCCGGGTCTG TCCGCCTAGT GCTTCAGCTC TTTAGCTCTG GTAGGACCGG TTGTACCACT

421 AACCCCATCT CTACTAAAAA TACAAAAATT AGCTGGGCGT GGTGGCGGGC TCCTGTAGTC
    TTGGGGTAGA GATGATTTTT ATGTTTTTAA TCGACCCGCA CCACCGCCCG AGGACATCAG

481 CCAGCTACTC AGGAGGCTGA GGCAGGAGAA TCGCTTGAAC CGGGGAGGCG GAGGTTGCAG
    GGTCGATGAG TCCTCCGACT CCGTCCTCTT AGCGAACTTG GCCCCTCCGC CTCCAACGTC

541 TCAGCCAAGA TAGCGCCACT GCACTGGAGC CTGGTGACAG AGTGAGACTC CCTCAAGAAA
    AGTCGGTTCT ATCGCGGTGA CGTGACCTCG GACCACTGTC TCACTCTGAG GGAGTTCTTT

601 GAAAGGAAGG GAAGGGAAAG GGAAGGAAGG GGAGGGGAAG GGAGGGGAGG GGAGGGGAGG
    CTTTCCTTCC CTTCCCTTTC CCTTCCTTCC CCTCCCCTTC CCTCCCCTCC CCTCCCCTCC

661 AAAGAAAAGA ATACTGGAAC TTGTTGAAGG CAGAGACTTT ATTTTCATAT CCCGGCTATG
    TTTCTTTTCT TATGACCTTG AACAACTTCC GTCTCTGAAA TAAAAGTATA GGGCCGATAC

721 TCTGGCTACT GTCTTACGTA ATAGATATAA AATCAATCTT GGTTGGATTA ACCAGAAGAA
    AGACCGATGA CAGAATGCAT TATCTATATT TTAGTTAGAA CCAACCTAAT TGGTCTTCTT
```

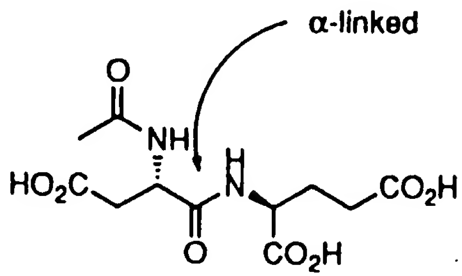
FIGURE 58B

```
781  TGAGAAGATA TATTCTGGTA AGTTGAATAC TTAGCACCCA GGGGTAATCA GCTTGGACAG
     ACTCTTCTAT ATAAGACCAT TCAACTTATG AATCGTGGGT CCCCATTAGT CGAACCTGTC

841  GACCAGGTCC AAAGACTGTT AAGAGTCTTC TGACTCCAAA CTCAGTGCTC CCTCCAGTGC
     CTGGTCCAGG TTTCTGACAA TTCTCAGAAG ACTGAGGTTT GAGTCACGAG GGAGGTCACG

901  CACAAGCAAA CTCCATAAAG GTATCCTGTG CTGAATAGAG ACTGTAGAGT GGTACAAAGT
     GTGTTCGTTT GAGGTATTTC CATAGGACAC GACTTATCTC TGACATCTCA CCATGTTTCA

961  AAGACAGACA TTATATTAAG TCTTAGCTTT GTGACTTCGA ATGACTTACC TAATCTAGCT
     TTCTGTCTGT AATATAATTC AGAATCGAAA CACTGAAGCT TACTGAATGG ATTAGATCGA

1021 AAATTTCAGT TTTACCATGT GTAAATCAGG AAGAGTAATA GAACAAACCT TGAAGGGTCC
     TTTAAAGTCA AAATGGTACA CATTTAGTCC TTCTCATTAT CTTGTTTGGA ACTTCCCAGG

1081 CAATGGTGAT TAAATGAGGT GATGTACATA ACATGCATCA CTCATAATAA GTGCTCTTTA
     GTTACCACTA ATTTACTCCA CTACATGTAT TGTACGTAGT GAGTATTATT CACGAGAAAT

1141 AATATTAGTC ACTATTATTA GCCATCTCTG ATTAGATTTG ACAATAGGAA CATTAGGAAA
     TTATAATCAG TGATAATAAT CGGTAGAGAC TAATCTAAAC TGTTATCCTT GTAATCCTTT

1201 GATATAGTAC ATTCAGGATT TTGTTAGAAA GAGATGAAGA AATTCCCTTC CTTCCTGCCC
     CTATATCATG TAAGTCCTAA AACAATCTTT CTCTACTTCT TTAAGGGAAG GAAGGACGGG

1261 TAGGTCATCT AGGAGTTGTC ATGGTTCATT GTTGACAAAT TAATTTTCCC AAATTTTTCA
     ATCCAGTAGA TCCTCAACAG TACCAAGTAA CAACTGTTTA ATTAAAAGGG TTTAAAAAGT

1321 CTTTGCTCAG AAAGTCTACA TCGAAGCACC CAAGACTGTA CAATCTAGTC CATCTTTTTC
     GAAACGAGTC TTTCAGATGT AGCTTCGTGG GTTCTGACAT GTTAGATCAG GTAGAAAAAG

1381 CACTTAACTC ATACTGTGCT CTCCCTTTCT CAAAGCAAAC TGTTTGCTAT TCCTTGAATA
     GTGAATTGAG TATGACACGA GAGGGAAAGA GTTTCGTTTG ACAAACGATA AGGAACTTAT

1441 CACTCTGAGT TTTCTGCCTT TGCCTACTCA GCTGGCCCAT GGCCCCTAAT GTTTCTTCTC
     GTGAGACTCA AAAGACGGAA ACGGATGAGT CGACCGGGTA CCGGGGATTA CAAAGAAGAG

1501 ATCTCCACTG GGTCAAATCC TACCTGTACC TTATGGTTCT GTTAAAAGCA GTGCTTCCAT
     TAGAGGTGAC CCAGTTTAGG ATGGACATGG AATACCAAGA CAATTTTCGT CACGAAGGTA

1561 AAAGTACTCC TAGCAAATGC ACGGCCTCTC TCACGGATTA TAAGAACACA GTTTATTTTA
     TTTCATGAGG ATCGTTTACG TGCCGGAGAG AGTGCCTAAT ATTCTTGTGT CAAATAAAAT

1621 TAAAGCATGT AGCTATTCTC TCCCTCGAAA TACGATTATT ATTATTAAGA ATTTATAGCA
     ATTTCGTACA TCGATAAGAG AGGGAGCTTT ATGCTAATAA TAATAATTCT TAAATATCGT

1681 GGGATATAAT TTTGTATGAT GATTCTTCTG GTTAATCCAA CCAAGATTGA TTTTATATCT
     CCCTATATTA AAACATACTA CTAAGAAGAC CAATTAGGTT GGTTCTAACT AAAATATAGA

1741 ATTACGTAAG ACAGTAGCCA GACATAGCCG GGATATGAAA ATAAAGTCTC TGCCTTCAAC
     TAATGCATTC TGTCATCGGT CTGTATCGGC CCTATACTTT TATTTCAGAG ACGGAAGTTG

1801 AAGTTCCAGT ATTCTTTTCT TTCCTCCCCT CCCCTCCCCT CCCTTCCCCT CCCCTTCCTT
     TTCAAGGTCA TAAGAAAAGA AAGGAGGGGA GGGGAGGGGA GGGAAGGGGA GGGGAAGGAA

1861 CCCTTTCCCT TCCCTTCCTT TCTTTCTTGA GGGAGTCTCA CTCTGTCACC AGGCTCCAGT
     GGGAAAGGGA AGGGAAGGAA AGAAAGAACT CCCTCAGAGT GAGACAGTGG TCCGAGGTCA
```

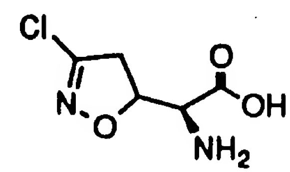
FIGURE 58C

```
1921 GCAGTGGCGC TATCTTGGCT GACTGCAACC TCCGCCTCCC CGGTTCAAGC GATTCTCCTG
     CGTCACCGCG ATAGAACCGA CTGACGTTGG AGGCGGAGGG GCCAAGTTCG CTAAGAGGAC

1981 CCTCAGCCTC CTGAGTAGCT GGGACTACAG GAGCCCGCCA CCACGCCCAG CTAATTTTTG
     GGAGTCGGAG GACTCATCGA CCCTGATGTC CTCGGGCGGT GGTGCGGGTC GATTAAAAAC

2041 TATTTTTAGT AGAGATGGGG TTTCACCATG TTGGCCAGGA TGGTCTCGAT TTCTCGACTT
     ATAAAAATCA TCTCTACCCC AAAGTGGTAC AACCGGTCCT ACCAGAGCTA AAGAGCTGAA

2101 CGTGATCCGC CTGTCTGGGC CTCCCAAAGT GCTGGGATTA CAGGCGTGAG CCACCACGCC
     GCACTAGGCG GACAGACCCG GAGGGTTTCA CGACCCTAAT GTCCGCACTC GGTGGTGCGG

2161 CGGCTTTAAA AAATGGTTTT GTAATGTAAG TGGAGGATAA TACCCTACAT GTTTATTAAT
     GCCGAAATTT TTTACCAAAA CATTACATTC ACCTCCTATT ATGGGATGTA CAAATAATTA

2221 AACAATAATA TTCTTTAGGA AAAAGGGCGC GGTGGTGATT TACACTGATG ACAAGCATTC
     TTGTTATTAT AAGAAATCCT TTTTCCCGCG CCACCACTAA ATGTGACTAC TGTTCGTAAG

2281 CCGACTATGG AAAAAAAGCG CAGCTTTTTC TGCTCTGCTT TTATTCAGTA GAGTATTGTA
     GGCTGATACC TTTTTTTCGC GTCGAAAAAG ACGAGACGAA AATAAGTCAT CTCATAACAT

2341 GAGATTGTAT AGAATTTCAG AGTTGAATAA AAGTTCCTCA TAATTATAGG AGTGGAGAGA
     CTCTAACATA TCTTAAAGTC TCAACTTATT TTCAAGGAGT ATTAATATCC TCACCTCTCT

2401 GGAGAGTCTC TTTCTTCCTT TCATTTTTAT ATTTAAGCAA GAGCTGGACA TTTTCCAAGA
     CCTCTCAGAG AAAGAAGGAA AGTAAAAATA TAAATTCGTT CTCGACCTGT AAAAGGTTCT

2461 AAGTTTTTTT TTTTTAAGGC GCCTCTCAAA AGGGGCCGGA TTTCCTTCTC CTGGAGGCAG
     TTCAAAAAAA AAAAATTCCG CGGAGAGTTT TCCCCGGCCT AAAGGAAGAG GACCTCCGTC

2521 ATGTTGCCTC TCTCTCTCGC TCGGATTGGT TCAGTGCACT CTAGAAACAC TGCTGTGGTG
     TACAACGGAG AGAGAGAGCG AGCCTAACCA AGTCACGTGA GATCTTTGTG ACGACACCAC

2581 GAGAAACTGG ACCCCAGGTC TGGAGCGAAT TCCAGCCTGC AGGGCTGATA AGCGAGGCAT
     CTCTTTGACC TGGGGTCCAG ACCTCGCTTA AGGTCGGACG TCCCGACTAT TCGCTCCGTA

2641 TAGTGAGATT GAGAGAGACT TTACCCCGCC GTGGTGGTTG GAGGGCGCGC AGTAGAGCAG
     ATCACTCTAA CTCTCTCTGA AATGGGGCGG CACCACCAAC CTCCCGCGCG TCATCTCGTC

2701 CAGCACAGGC GCGGGTCCCG GGAGGCCGGC TCTGCTCGCG CCGAGATGTG GAATCTCCTT
     GTCGTGTCCG CGCCCAGGGC CCTCCGGCCG AGACGAGCGC GGCTCTACAC CTTAGAGGAA

2761 CACGAAACCG ACTCGGCTGT GGCCACCGCC CGCCGCCCGC GCTGGCTGTG CGCTGGGGCG
     GTGCTTTGGC TGAGCCGACA CCGGTGGCGG GCGGCGGGCG CGACCGACAC GCGACCCCGC

2821 CTGGTGCTGG CGGGTGGCTT CTTTCTCCTC GGCTTCCTCT TCGGTAGGGG GGCGCCTCGC
     GACCACGACC GCCCACCGAA GAAAGAGGAG CCGAAGGAGA AGCCATCCCC CCGCGGAGCG

2881 GGAGCAAACC TCGGAGTCTT CCCCGTGGTG CCGCGGTGCT GGGACTCGCG GGTCAGCTGC
     CCTCGTTTGG AGCCTCAGAA GGGGCACCAC GGCGCCACGA CCCTGAGCGC CCAGTCGACG

2941 CGAGTGGGAT CCTGTTGCTG GTCTTCCCCA GGGGCGGCGA TTAGGGTCGG GGTAATGTGG
     GCTCACCCTA GGACAACGAC CAGAAGGGGT CCCCGCCGCT AATCCCAGCC CCATTACACC

3001 GGTGAGCACC CCTCGAG
     CCACTCGTGG GGAGCTC
```

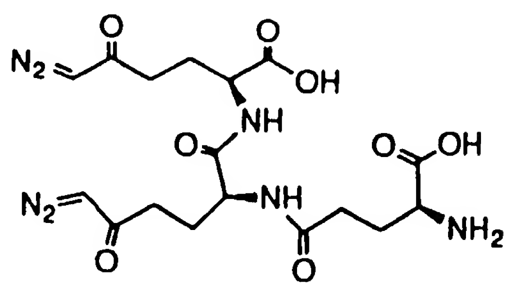
FIG. 59



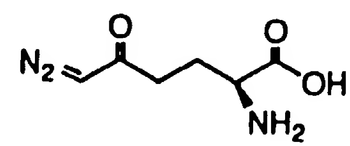
NAAG 1
N-acetylaspartyl-L-glutamate



Acivicin

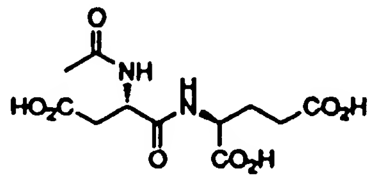


Azotomycin, becomes active by *in vivo* conversion to DON

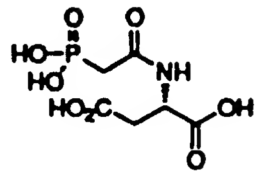


6-diazo-5-oxo-norleucine, DON

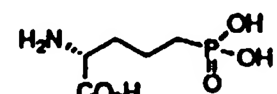
FIG. 60



NAAG 1
N-acetylaspartyl-L-glutamate

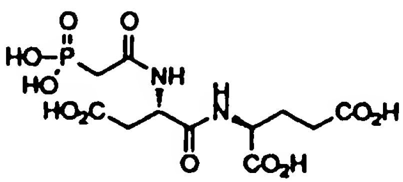


PALA 2
N-phosphonoacetyl-L-aspartate

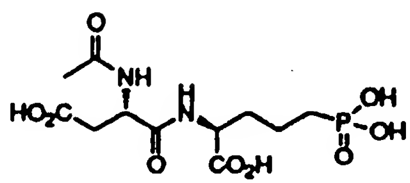


D-APV

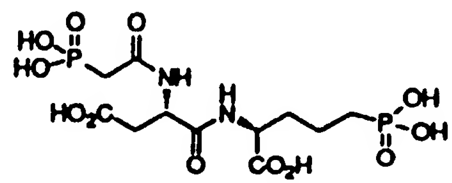
L-Glu antagonist



3

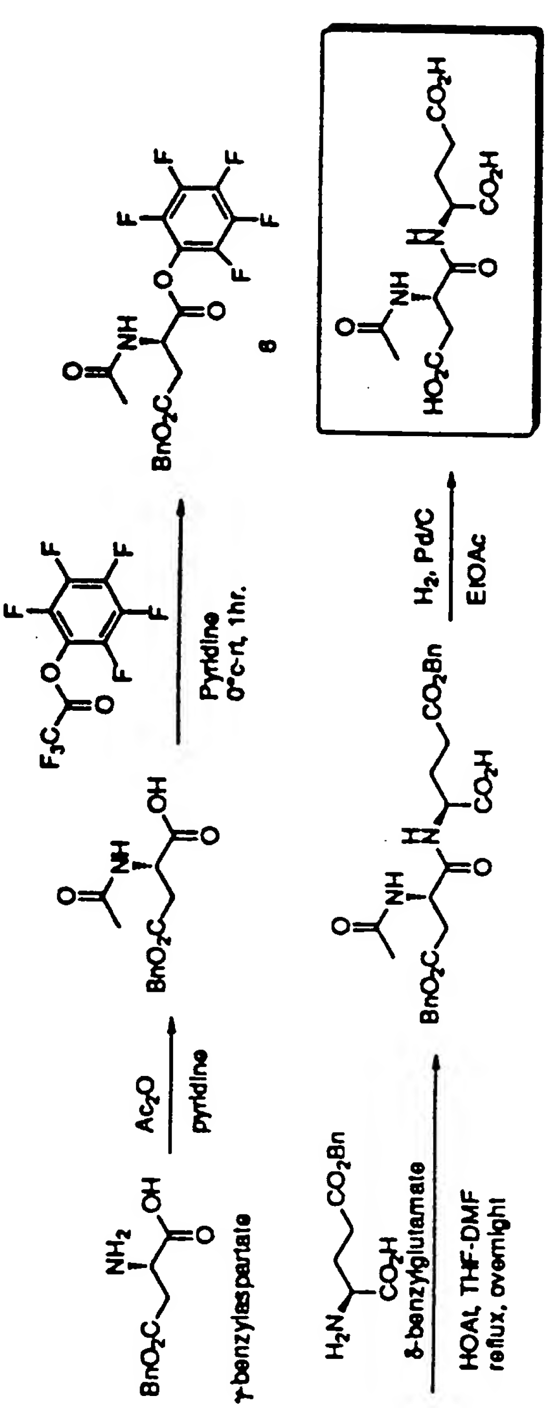


4



5

FIG. 61



NAAG 1
Identical in all respects to an authentic sample from Sigma.

Ac_2O = acetic anhydride
THF = tetrahydrofurane
DMF = N,N-dimethylformamide
Pd/C = palladium on charcoal
EtOAc = ethylacetate

HOAt =

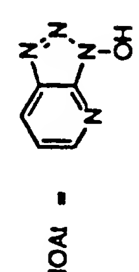


FIG. 62

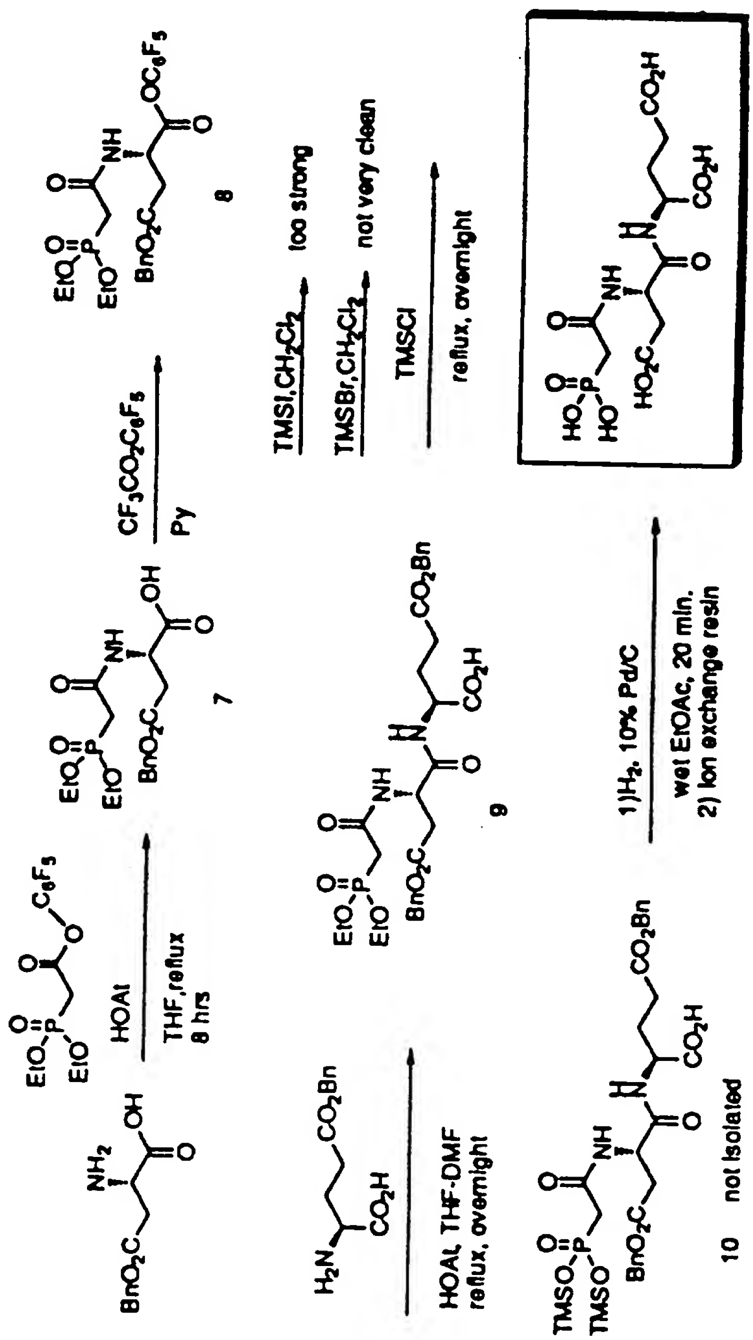

HOAt
THF, reflux
8 hrs
7
$CF_3CO_2C_6F_5$
Py
8
HOAt, THF-DMF
reflux, overnight
9
TMSI, CH_2Cl_2
too strong
TMSBr, CH_2Cl_2
not very clean
TMSCl
reflux, overnight
10 not isolated
1) H_2, 10% Pd/C
wet EtOAc, 20 min.
2) Ion exchange resin
PALA-Glu, 3

FIG. 63

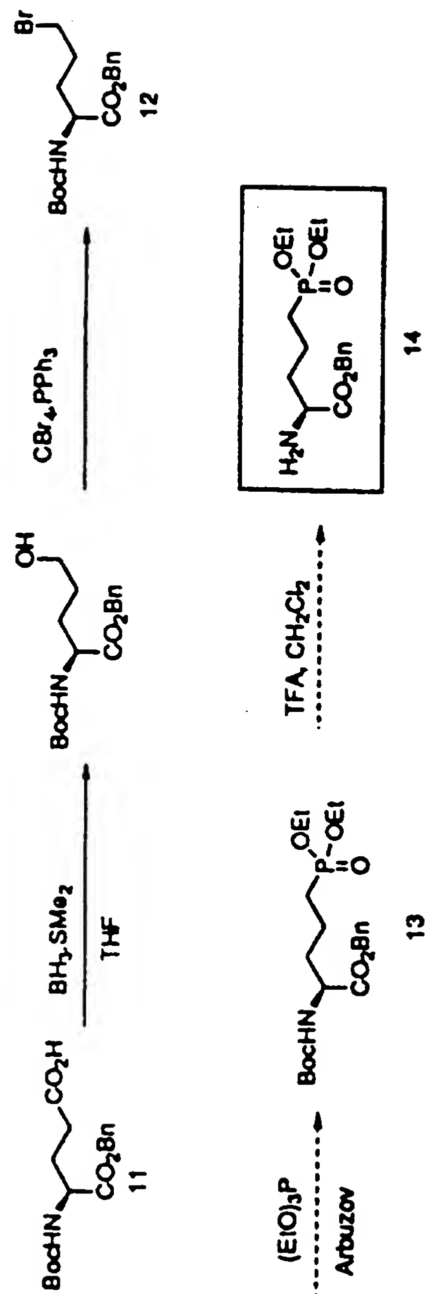

BocHN
CO2H
CO2Bn
11
BH3.SMe2
THF
OH
CBr4,PPh3
Br
12
(EtO)3P
Arbuzov
OEt
13
TFA, CH2Cl2
H2N
14

FIG. 64

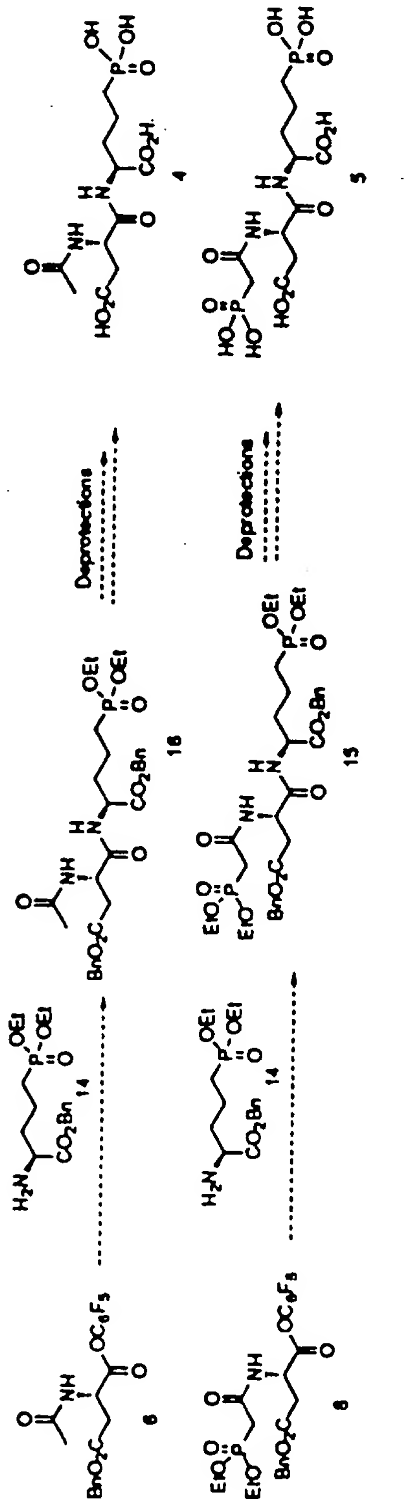
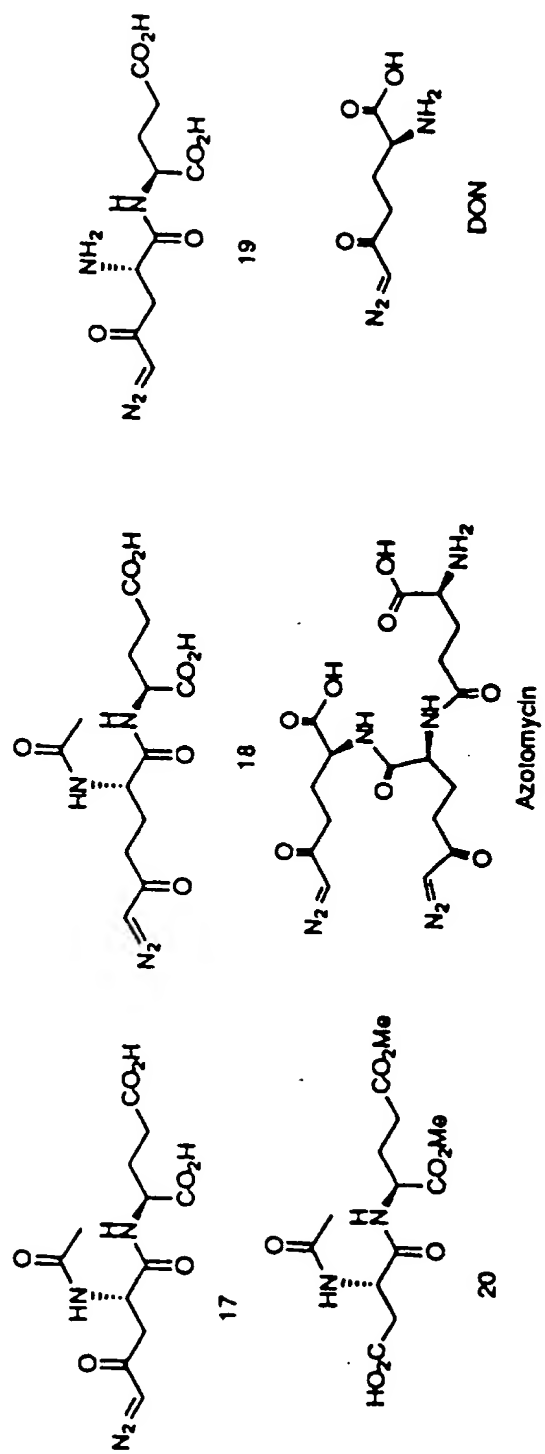


FIG. 65



19

DON

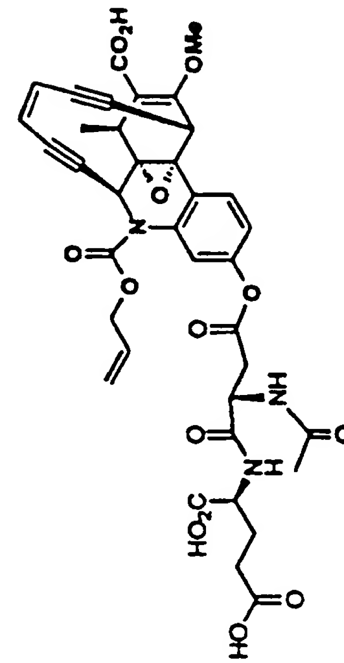
18

Azotomycin

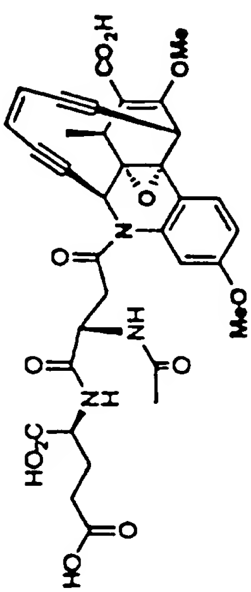
17

20

FIG. 66



22



21

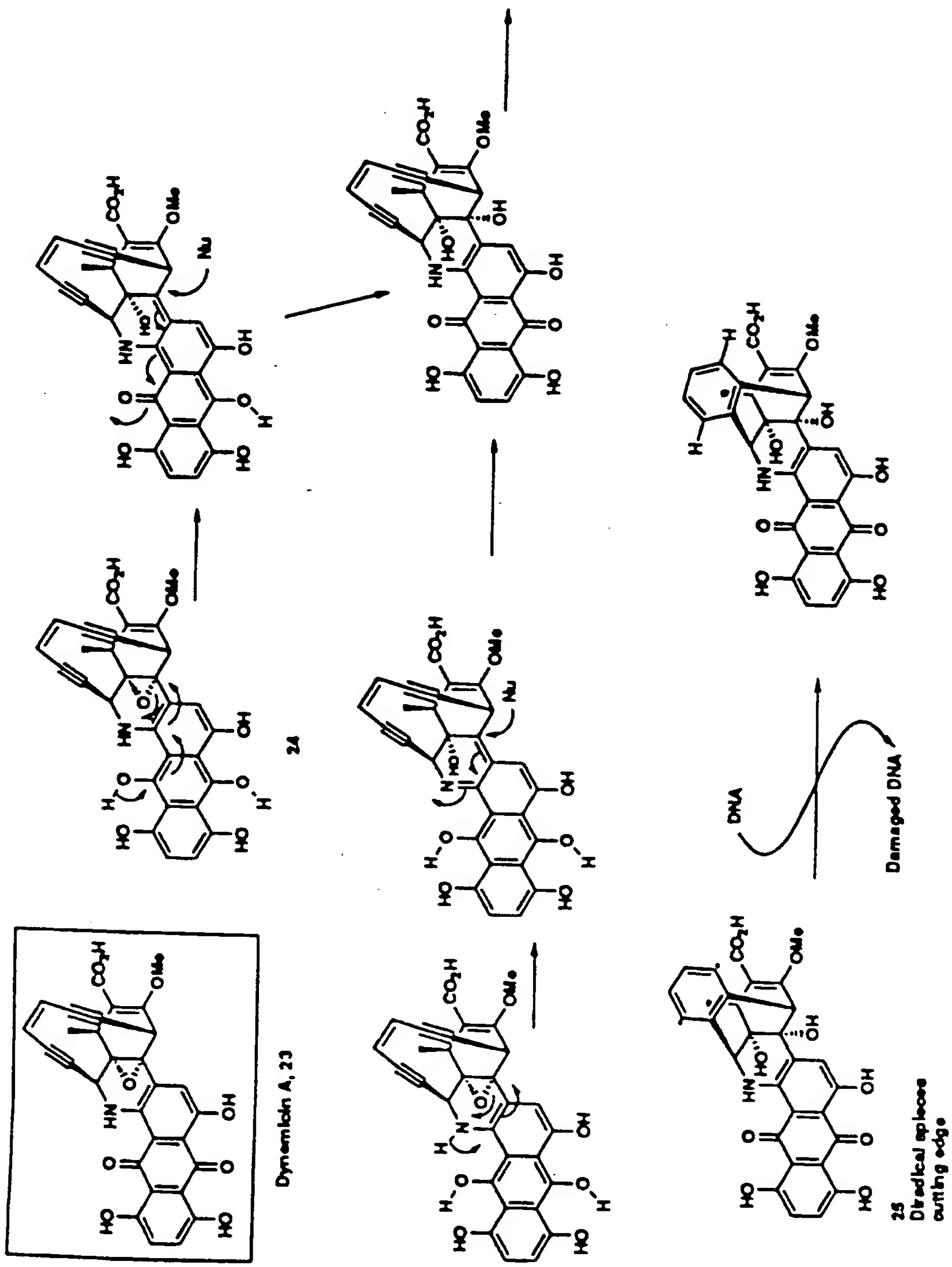
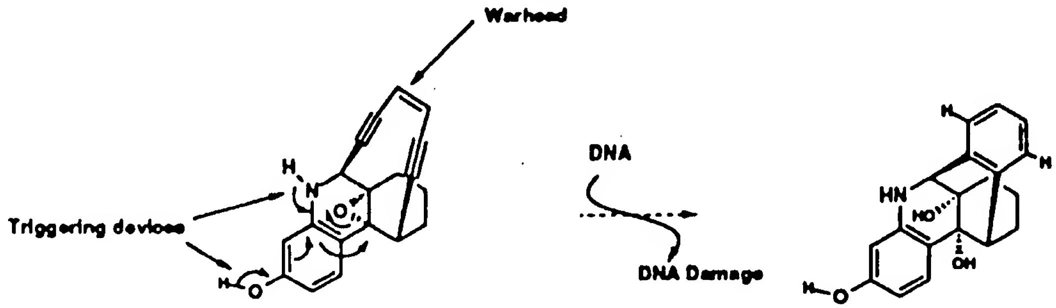
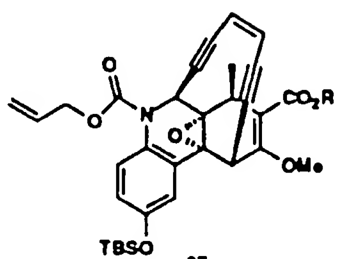

Dynemicin A, 23
24
25
Diradical splicose cutting edge
DNA
Damaged DNA
Nu

FIG. 67

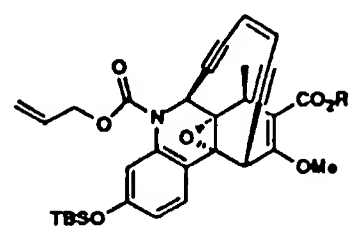
FIG. 68



26
active at the nano to picomolar levels in different cell lines
readily rearranges when one or both
triggering devices are deprotected

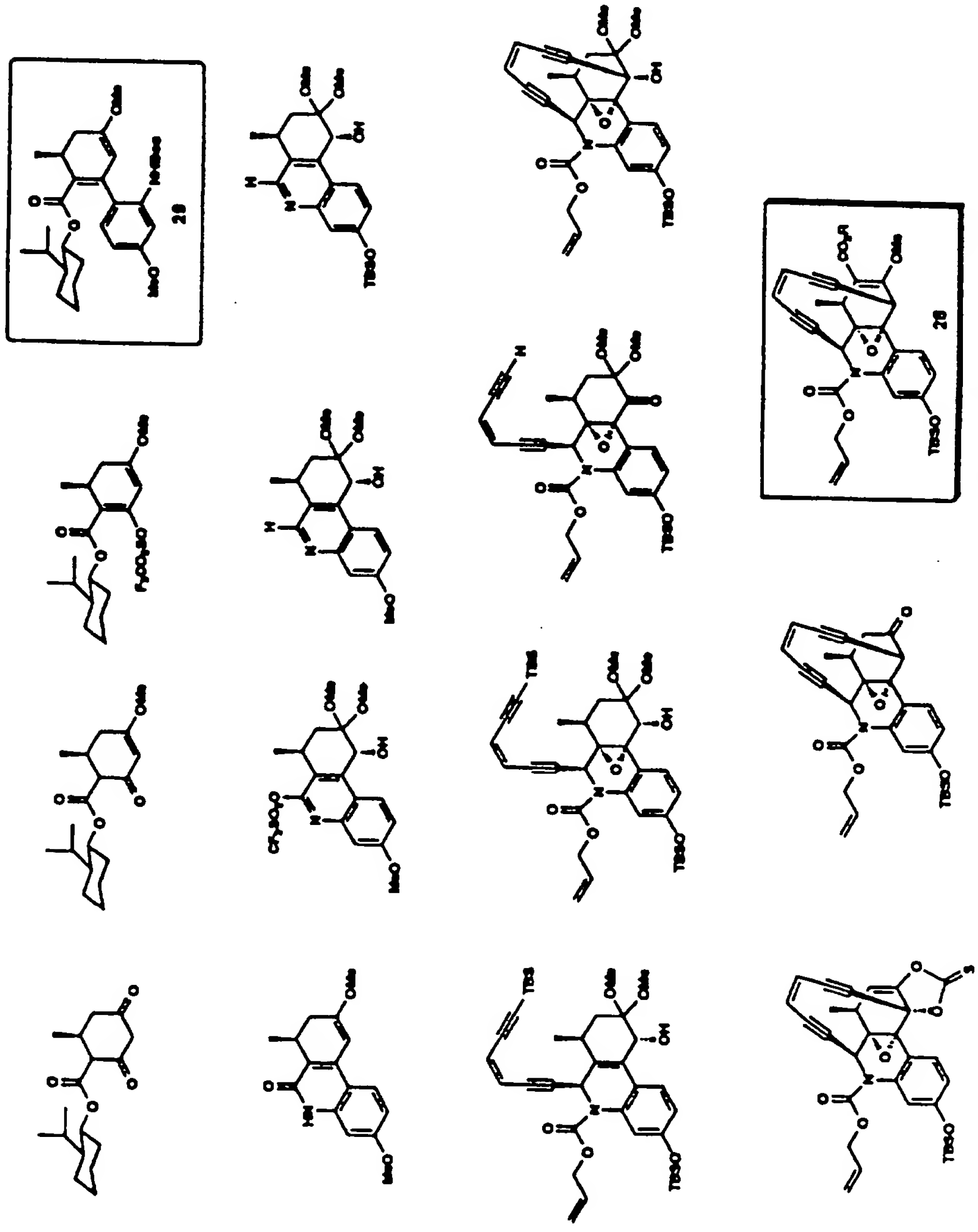


27



28

FIG. 69



17 Steps for the optically active form 1

FIG. 70

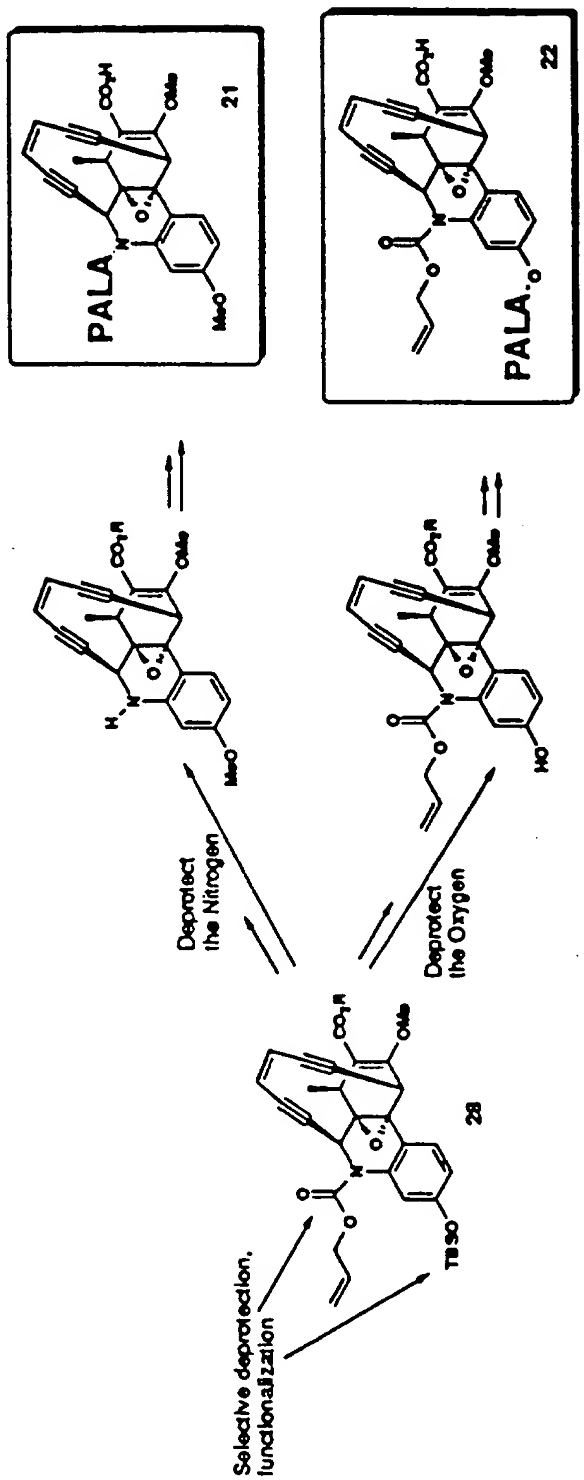

Selective deprotection, functionalization
TBSO
28
CO_2R
OMe
Deprotect the Nitrogen
Deprotect the Oxygen
MeO
HO
PALA
CO_2H
21
22

FIG. 71

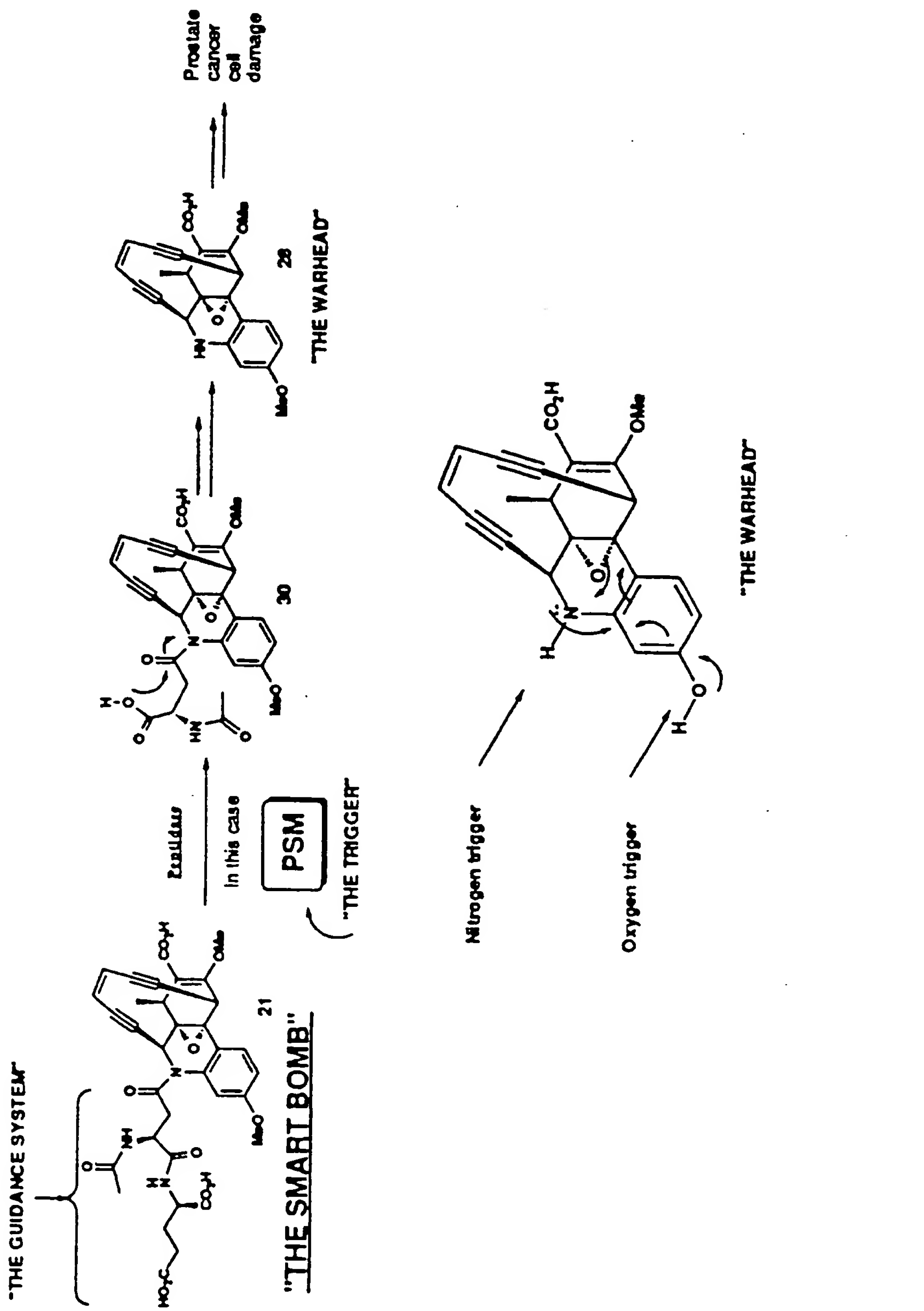

"THE GUIDANCE SYSTEM"
"THE SMART BOMB"
21
Peptidase
In this case
PSM
"THE TRIGGER"
30
28
"THE WARHEAD"
Prostate cancer cell damage
Nitrogen trigger
Oxygen trigger
"THE WARHEAD"

FIG. 72A

```
              10         20         30         40         50         60
               |          |          |          |          |          |
  1  TAGGGGGGCG CCTCGCGGAG AAACCTCGGA GTCTTCCCCG TGGTGCCGCG GTGCTGGGAC
     ATCCCCCCGC GGAGCGCCTC TTTGGAGCCT CAGAAGGGGC ACCACGGCGC CACGACCCTG

 61  TCGCGGGTCA GCTGCCGAGT GGGATCCTGT TGCTGGTCTT CCCCAGGGGC GGCGATTAGG
     AGCGCCCAGT CGACGGCTCA CCCTAGGACA ACGACCAGAA GGGGTCCCCG CCGCTAATCC

121  GTCGGGGTAA TGTGGGGTGA GCACCCCTCG AGTTAGGAGG AGGGTAGCTG GGAACGGTGC
     CAGCCCCATT ACACCCCACT CGTGGGGAGC TCAATCCTCC TCCCATCGAC CCTTGCCACG

181  AGGGCTGAGT TCTCGACAAG CTGCTGGTAG GACAGTCACT CAGGTTGAGG GTAGAACTGA
     TCCCGACTCA AGAGCTGTTC GACGACCATC CTGTCAGTGA GTCCAACTCC CATCTTGACT

241  GAGAACCTGA AACTGGGCGT AGGAAGGTTC CAAGTGCTGG AGCCCTGCAA GACAGAGGAA
     CTCTTGGACT TTGACCCGCA TCCTTCCAAG GTTCACGACC TCGGGACGTT CTGTCTCCTT

301  GTTTTTTTTT TGCTTTTGTT TTGTTTTGTT TTGTTTTGTT TTGTTTTGTT TGTTTGTTTG
     CAAAAAAAAA ACGAAAACAA AACAAAACAA AACAAAACAA AACAAAACAA ACAAACAAAC

361  TTTTTTTACC TCTCTGTGCA TTCTTTCTTC CTTGGAAGTA ACAGAGGCAA GCTTGGGAAC
     AAAAAAATGG AGAGACACGT AAGAAAGAAG GAACCTTCAT TGTCTCCGTT CGAACCCTTG

421  TGTGTGAACC AGGTCAGCAA TCTGGACAGG TCTTTACCAG CGGGTCTTTT GCTGTTTTTC
     ACACACTTGG TCCAGTCGTT AGACCTGTCC AGAAATGGTC GCCCAGAAAA CGACAAAAAG

481  CTGGGTACTG ATTTGCAGAC TTGATCCAAC TTTCTAAGAA AAGCAGAACC ACACAGGCAA
     GACCCATGAC TAAACGTCTG AACTAGGTTG AAAGATTCTT TTCGTCTTGG TGTGTCCGTT

541  GCTCAGACTC TTTTATTAAA TTCCAGTTTT GACTTTGCCA CTTCTTAGTG GCCTTGAACA
     CGAGTCTGAG AAAATAATTT AAGGTCAAAA CTGAAACGGT GAAGAATCAC CGGAACTTGT
```

FIG. 72B

```
601 AGTTACCGAG TCCCTCTCAG CGTTAGTTAC CCTATTTTAT GATGAGGATA ATATTATCTG
    TCAATGGCTC AGGGAGAGTC GCAATCAATG GGATAAAATA CTACTCCTAT TATAATAGAC

661 CAAATTATTG GTAATAGTAA ATAATATAGC ATGTAAATCT CCTAGCACAG TACTGGGATT
    GTTTAATAAC CATTATCATT TATTATATCG TACATTTAGA GGATCGTGTC ATGACCCTAA

721 TTCGCCACTT TATTTCTTCT TTTACCAAGA TACTCCTCAT TGGACTTTAA TACACAGGAC
    AAGCGGTGAA ATAAAGAAGA AAATGGTTCT ATGAGGAGTA ACCTGAAATT ATGTGTCCTG

781 TAGTCTAAGG TATCACCAGG TAGTCCACTC CTGCTCGGAA TTCTTGACCC TCTTTCGGGA
    ATCAGATTCC ATAGTGGTCC ATCAGGTGAG GACGAGCCTT AAGAACTGGG AGAAAGCCCT

841 TTTAGAAGAA TAGGGCATGG ACCAGATGGG TTTAAACAAA TTCAATATCT TCCACTAGCT
    AAATCTTCTT ATCCCGTACC TGGTCTACCC AAATTTGTTT AAGTTATAGA AGGTGATCGA

901 TCACCTTGGG GTTGTTAAAA GATTTTTGAA CCACACACTG TGCTCATAAC AATCTTCATC
    AGTGGAACCC CAACAATTTT CTAAAAACTT GGTGTGTGAC ACGAGTATTG TTAGAAGTAG

961 TCTTAAAAGG ATTTTATTCT TCCTGGTATT GCCCTCACTC TCATCCCTGT ATTCCGTGCT
    AGAATTTTCC TAAAATAAGA AGGACCATAA CGGGAGTGAG AGTAGGGACA TAAGGCACGA
```

FIG. 72C

```
1021 CAGTGGCTGA CACAGAAGAG TTCTTTATTG ATGTCCGCCC CCCACCCACT AGGATTCTCT
     GTCACCGACT GTGTCTTCTC AAGAAATAAC TACAGGCGGG GGGTGGGTGA TCCTAAGAGA

1081 GCTCTCCCCT CCCCCTACAG GCCTCCATCC TCTTCATCCT GTTCATTTTT CAGATCTCAG
     CGAGAGGGGA GGGGGATGTC CGGAGGTAGG AGAAGTAGGA CAAGTAAAAA GTCTAGAGTC

1141 TTCAAGCATC TCGTCCTCAG TGTGGTGTTT CCTGATCCCT CACTCTAATC CAAGTCTTTC
     AAGTTCGTAG AGCAGGAGTC ACACCACAAA GGACTAGGGA GTGAGATTAG GTTCAGAAAG

1201 TGTTTTATGC ACAGGTGGAA TCTTATTTCC GTTTGCGTCC AATCATGTAT TTTAATATGC
     ACAAAATACG TGTCCACCTT AGAATAAAGG CAAACGCAGG TTAGTACATA AAATTATACG

1261 ATGTATATAT GTATCTGCAT TTGTATGCAT GCGATTAAGA ACTAGAATAA TTAATAATTG
     TACATATATA CATACACGTA AACATACGTA CGCTAATTCT TGATCTTATT AATTATTAAC

1321 GAAAGCTCCA TGAAAGCTGG TTGGGGACTA ATTTTGTAAC TACTTTATTC CCAGATCCTG
     CTTTCGAGGT ACTTTCGACC AACCCCTGAT TAAAACATTG ATGAAATAAG GGTCTAGGAC

1381 TAATTTCTCT AAATAAACCC TGGAATCTTG CCTTATCTCC TTCAGGTTAA AAGCCAACTG
     ATTAAAGAGA TTTATTTGGG ACCTTAGAAC GGAATAGAGG AAGTCCAATT TTCGGTTGAC

1441 CAAGGTCTAA TGACTGCAGG ATCTAGCTAT CCATTGTTTC TGGCCGCCTA TGCGTGCACT
     GTTCCAGATT ACTGACGTCC TAGATCGATA GGTAACAAAG ACCGGCGGAT ACGCACGTGA

1501 GGGTGTCTGG CAGAGAGGCT GGGTAAATTG TAGTTTCATT GTAGCTGTCT GACTTGGATT
     CCCACAGACC GTCTCTCCGA CCCATTTAAC ATCAAAGTAA CATCGACAGA CTGAACCTAA

1561 TCTCACGCCT ACTTCACTGG AAACGCAAAC TCTCACAGCA TTTTGTTTTA GTTTCAGAAT
     AGAGTGCGGA TGAAGTGACC TTTGCGTTTG AGAGTGTCGT AAAACAAAAT CAAAGTCTTA

1621 CAGAGCAAAT TAGAAGTCTG AATTTCCTTC AACACTTGGA AATAATTTAT TTATTTGAAA
     GTCTCGTTTA ATCTTCAGAC TTAAAGGAAG TTGTGAACCT TTATTAAATA AATAAACTTT

1681 TATATTCATA ATTAATTCGT TATAAAAATG TATTAAATGC TTATTTGAGT CAGCAGAGGA
     ATATAAGTAT TAATTAAGCA ATATTTTTAC ATAATTTACG AATAAACTCA GTCGTCTCCT
```

FIG. 72D

```
1741 AGATAGAAAC TTTATGAAAG TAGAAGGTGG ATCTCCTTTT TGCCTTCATT TTCAGAACAT
     TCTATCTTTG AAATACTTTC ATCTTCCACC TAGAGGAAAA ACGGAAGTAA AAGTCTTGTA

1801 CTCGTTTACA CCCATTAGTT GAAACATTAA TGTCATTTTA TTTTCGTCCT GATTATCTCA
     GAGCAAATGT GGGTAATCAA CTTTGTAATT ACAGTAAAAT AAAAGCAGGA CTAATAGAGT

1861 TAAAACATTT CTTAGAATAA CAGCAATACC TATCATTGAA GTTGGATAAG AAATATTTTG
     ATTTTGTAAA GAATCTTATT GTCGTTATGG ATAGTAACTT CAACCTATTC TTTATAAAAC

1921 CAATTGGTTT GCAACTTAAA AATCTGTTTG CATGACTCTT TTTCAGTGAA AGTAGGCAAG
     GTTAACCAAA CGTTGAATTT TTAGACAAAC GTACTGAGAA AAAGTCACTT TCATCCGTTC

1981 AGAAATTAAA ATTCAGAAAT ATCTCACCTA ATGTCAGAGG TAATATTGAT AATTTGTGTT
     TCTTTAATTT TAAGTCTTTA TAGAGTGGAT TACAGTCTCC ATTATAACTA TTAAACACAA

2041 TTACAAATAA TACATACAAC AATAATGAAA AATAAGTCCT ATCTATAGGC TCGTATCTCA
     AATGTTTATT ATGTATGTTG TTATTACTTT TTATTCAGGA TAGATATCCG AGCATAGAGT

2101 TGCCTATTTT TGGATGTATT TTTCA
     ACGGATAAAA ACCTACATAA AAAGT
```

FIG. 73A

```
                10         20         30         40         50         60
                 |          |          |          |          |          |
  1  TGAAAAATAC ATCAAAAATA GGCATGAGAT ACGAGCCTAT AGATAGGACT TATTTTTTAT
     ACTTTTTATG TAGTTTTTAT CCGTACTCTA TGCTCGGATA TCTATCCTGA ATAAAAAATA

 61  TATTGTTGTA TGTATTATTT GTAAAACACA AATTATCAAT ATTACCTCTG ACATTAGGTG
     ATAACAACAT ACATAATAAA CATTTTGTGT TTAATAGTTA TAATGGAGAC TGTAATCCAC

121  AGATATTCTG AATTTTAATT TCTCTTGCCT ACTTTCACTG AAAAAGAGTC ATGCAAACAG
     TCTATAAGAC TTAAAATTAA AGAGAACGGA TGAAAGTGAC TTTTTCTCAG TACGTTTGTC

181  ATTTTTAAGT TGCAAACCAA TTGCAAAATA TTTTTTTATC CAACTTCAAT GATAGGTATT
     TAAAAATTCA ACGTTTGGTT AACGTTTTAT AAAAAAATAG GTTGAAGTTA CTATCCATAA

241  GCTGTTAATT CTAAGATATG CATTAATTGT TTCAACTAAT GGGTGTCAAA CGAGATGTTC
     CGACAATTAA GATTCTATAC GTAATTAACA AAGTTGATTA CCCACAGTTT GCTCTACAAG

301  TGAAAATGAA GGCAAAAAGG AGATCCACCT TCTACTTTCA TAAAGTTTCT ATCTTCCTCT
     ACTTTTACTT CCGTTTTTCC TCTAGGTGGA AGATGAAAGT ATTTCAAAGA TAGAAGGAGA

361  GCTGACTCAA ATAAGCATTT AATACATTTT ATAACGAATT AATTATGAAT ATATTTCAAA
     CGACTGAGTT TATTCGTAAA TTATGTAAAA TATTGCTTAA TTAATACTTA TATAAAGTTT

421  TAAATAAATT ATTTCCAAGT GTTGAAGGAA ATTCAGACTT CTAATTTGCT CTGATTCTGA
     ATTTATTTAA TAAAGGTTCA CAACTTCCTT TAAGTCTGAA GATTAAACGA GACTAAGACT
```

FIG. 73B

```
481 AACTAAAACA AATGCTCTGT GAGAGTTTGC GTTTCCAGTG AAGTAGCGTG AGAAATCCAA
    TTGATTTTGT TTACGAGACA CTCTCAAACG CAAAGGTCAC TTCATCGCAC TCTTTAGGTT

541 GTCAGACAGC TACATGAAAC TACATTTACC AGCTCTCTGC CAGACACCAG TGCACGATAG
    CAGTCTGTCG ATGTACTTTG ATGTAAATGG TCGAGAGACG GTCTGTGGTC ACGTGCTATC

601 CGCAGAACAT GTAGCTAGAT CTCAGTCATA GCTNNNNNNN NNNNNNNNNN AGACCTTGCA
    GCGTCTTGTA CATCGATCTA GAGTCAGTAT CGANNNNNNN NNNNNNNNNN TCTGGAACGT

661 GTTGGCTTTT AACCTGAAGG AGATAAGGCA AGATTCCAGG GTTTATTTAG AGAAATTACA
    CAACCGAAAA TTGGACTTCC TCTATTCCGT TCTAAGGTCC CAAATAAATC TCTTTAATGT

721 GGATCTGGGA ATAAAGTAGT TACAAAATTA GTCCCCAACC AGCTTTCATG GAGCTTTCAA
    CCTAGACCCT TATTTCATCA ATGTTTTAAT CAGGGGTTGG TCGAAAGTAC CTCGAAAGTT
```

FIG. 73C

```
 781 TTATTAATTA TTCTAGTTCT TAATCGCATG CATACAATGC ACATACATAT ATACATGCAT
     AATAATTAAT AAGATCAAGA ATTAGCGTAC GTATGTTACG TGTATGTATA TATGTACGTA

 841 ATTAAAATAC ATGATTGGAC GCAAACGGAA ATAAGATTCC ACCTGTGCAT AAAACAGAAA
     TAATTTTATG TACTAACCTG CGTTTGCCTT TATTCTAAGG TGGACACGTA TTTTGTCTTT

 901 GACTTGGTTA GAGTGAGGGA TCAGGAAACA CCACACTGAG GACGAGATGN NNNNNNNNNN
     CTGAACCAAT CTCACTCCCT AGTCCTTTGT GGTGTGACTC CTGCTCTACN NNNNNNNNNN

 961 NTAGTGGGTG GGGGGCGGAC ATCAATAAAG AACTCTTCTG TGTCAGCCAC TGAGCACGGA
     NATCACCCAC CCCCCGCCTG TAGTTATTTC TTGAGAAGAC ACAGTCGGTG ACTCGTGCCT

1021 ATAAAGGGAT GAGAGTGAGG GCAANTACCA GAAGAATAAA ATCCTTTTAA GAGATGAAGA
     TATTTCCCTA CTCTCACTCC CGTTNATGGT CTTCTTATTT TAGGAAAATT CTCTACTTCT

1081 TTGTTATGAG CACAGTGTGT GGNTTCAAAA ATCTTTTAAC AACCCCAAGG TGAAGCTAGT
     AACAATACTC GTGTCACACA CCNAAGTTTT TAGAAAATTG TTGGGGTTCC ACTTCGATCA

1141 TGGAAGATAT TTGAATTTGT TTAAACCCAT CTGGTCCTAG CCCTATTCTT TGAATCCCGA
     ACCTTCTATA AACTTAAACA AATTTGGGTA GACCAGGATC GGGATAAGAA ACTTAGGGCT
```

FIG. 73D

```
1201 AAGAGGGTCA AGAATTCCGA GCAGGAGTGG ACTACCTGGT GATACCTTAG ACTAGTCCTG
     TTCTCCCAGT TCTTAAGGCT CGTCCTCACC TGATGGACCA CTATGGAATC TGATCAGGAC

1261 TGTATTAAAG TCCAATGAGG AGTATCTTGG TAAAATAATA AATAAAGTCC CGAAAATCCC
     ACATAATTTC AGGTTACTCC TCATAGAACC ATTTTATTAT TTATTTCAGG GCTTTTAGGG

1321 AGTACTGTGC TAGGAGATTT ACATGCTATA TTATTTACTA TNNNNNNNNT AATTTGCAGA
     TCATGACACG ATCCTCTAAA TGTACGATAT AATAAATGAT ANNNNNNNNA TTAAACGTCT

1381 TAATATTATC CTCATCATAA AATAGGGTAA CTAACGCTGA GAGGGACTCG GTAACTTGTT
     ATTATAATAG GAGTAGTATT TTATCCCATT GATTGCGACT CTCCCTGAGC CATTGAACAA

1441 CAAGGCCACT AAGAAGTGGC AAAGTCAAAA CTGGAATTTT AATAAAAGAG TCTAGCTTGC
     GTTCCGGTGA TTCTTCACCG TTTCAGTTTT GACCTTAAAA TTATTTTCTC AGATCGAACG

1501 CTGTGTGGTT CTGCTTTTCT TAGAAAGTTG GANNAAGTCT CANATCAGTA CCCAGGAAAA
     GACACACCAA GACGAAAAGA ATCTTTCAAC CTNNTTCAGA GTNTAGTCAT GGGTCCTTTT

1561 ACAGCAAAAG ACCCGCTGGT AAAGACCTGT CCAGATTGCT GACCTGGTTC ACACANNTCC
```

FIG. 73E

```
     TGTCGTTTTC TGGGCGACCA TTTCTGGACA GGTCTAACGA CTGGACCAAG TGTGTNNAGG

1621 AAGCTTGCCT CTGTTACTTC CAAGGAAGAA AGAATGCACA GAGAGGTAAA AAAACAAACA
     TTCGAACGGA GACAATGAAG GTTCCTTCTT TCTTACGTGT CTCTCCATTT TTTTGTTTGT

1681 AACCAAACAA AACAAAACAA AACAAAACAA AACAAAACAA AAGCAAAAAA AAACTTCCTC
     TTGGTTTGTT TTGTTTTGTT TTGTTTTGTT TTGTTTTGTT TTCGTTTTTT TTTGAAGGAG

1741 TGTCTTGCAG GGCTCCAGCA CTTGGAACCT TCCTACGTCC TANTTTCAGG TTCTCTCAGT
     ACAGAACGTC CCGAGGTCGT GAACCTTGGA AGGATGCAGG ATNAAAGTCC AAGAGAGTCA

1801 TCTACCCTCA ACCTGAGTGA CTGTCCTACC AGCAGCTTGT CGAGAACTCA GCCCTGCACC
     AGATGGGAGT TGGACTCACT GACAGGATGG TCGTCGAACA GCTCTTGAGT CGGGACGTGG

1861 GTTCCCAGCT ACCCTCCTCC TAACTCGAGG GGTGCT
     CAAGGGTCGA TGGGAGGAGG ATTGAGCTCC CCACGA
```

FIG. 74A

```
                10         20         30         40         50         60
                 |          |          |          |          |          |
  1 GGATTCTGTT GAGCCCTAGC TCATTATGAT GTCCTGTTGT CCTACCCAAA TAAGACTCAT
    CCTAAGACAA CTCGGGATCG AGTAATACTA CAGGACAACA GGATGGGTTT ATTCTGAGTA

 61 CCCAACTACA TCTCAATAAT TAATGAAGAT GGAAATGAGG TAAAAAATAA ATAAATAAAT
    GGGTTGATGT AGAGTTATTA ATTACTTCTA CCTTTACTCC ATTTTTTATT TATTTATTTA

121 AAAAGAAACA TTCCCCCCCA TTTATTATTT TTTCAAATAC CTTCTATGAA ATAATGTTCT
    TTTTCTTTGT AAGGGGGGGT AAATAATAAA AAAGTTTATG GAAGATACTT TATTACAAGA

181 ATCCCTCTCT AAATATTAAT AGAAATCAAT ATTATTGGAA CTGTGAATAC CTTTAATATC
    TAGGGAGAGA TTTATAATTA TCTTTAGTTA TAATAACCTT GACACTTATG GAAATTATAG

241 TCATTATCCG GTGTCAACTA CTTTCCTATG ATGTTGAGTT ACTGGGTTTA GAAGTCGGGA
    AGTAATAGGC CACAGTTGAT GAAAGGATAC TACAACTCAA TGACCCAAAT CTTCAGCCCT

301 AATAATGCTG TAAANNNNNN AGTTAGTCTA CACACCAATA TCAAATATGA TATACTTGTA
    TTATTACGAC ATTTNNNNNN TCAATCAGAT GTGTGGTTAT AGTTTATACT ATATGAACAT

361 AACCTCCAAG CATAAAAAGA GATACTTTAT AAAAGAGGTT CTTTTTTTCT TTTTTTTTTT
    TTGGAGGTTC GTATTTTTCT CTATGAAATA TTTTCTCCAA GAAAAAAAGA AAAAAAAAAA
```

FIG. 74B

```
421 TCCAGATGGA GTTTCACTCC TGTCAGGCAG GCNGAGTGCA GTGGTGCCAT CTCGGCTCAC
    AGGTCTACCT CAAAGTGAGG ACAGTCCGTC CGNCTCACGT CACCACGGTA GAGCCGAGTG

481 TGCAACCTCC ACCTCCCATG TTCAAGGGAT TCTCCTTCCT CAGTCTCCTG AGTAGCTGGG
    ACGTTGGAGG TGGAGGGTAC AAGTTCCCTA AGAGGAAGGA GTCAGAGGAC TCATCGACCC

541 ATTACAGGTG TGCACCACCA CACCCAGCTA ATTTTTGTAT TTTTAATAGA GACAGGGTTT
    TAATGTCCAC ACGTGGTGGT GTGGGTCGAT TAAAAACATA AAAATTATCT CTGTCCCAAA

601 CATCGATGTT GGCCAGGCTA GTCTCGAACT CCTGACCTCT AGGTGATCCA CCCGCCTCAG
    GTAGCTACAA CCGGTCCGAT CAGAGCTTGA GGACTGGAGA TCCACTAGGT GGGCGGAGTC

661 CCTCCCAAAG TTGTAGAATT ACACGTGTGA GGCACTGCTC TGGCCAGGAG ATACATTTTT
    GGAGGGTTTC AACATCTTAA TGTGCACACT CCGTGACGAG ACCGGTCCTC TATGTAAAAA

721 GATAGGTTTA ATTTATAAAG ACACTGCACA GATTTGGACT TGCTGGGAAA TCACGATCCA
    CTATCCAAAT TAAATATTTC TGTGACGTGT CTAAACCTCA ACGACCCTTT AGTGCTAGGT
```

FIG. 74C

```
781  GTATGCATTT GACCCAGCAA TTTTTATTGG TACTTAATGA TTATATCTCA ATTGATCAGG
     CATACGTAAA CTGGGTCGTT AAAAATAACC ATGAATTACT AATATAGAGT TAACTAGTCC

841  TTGAACTCTG TGCGAAGAAT TTGTGTGTGG ACATTTGAGA GGACAGTTTG GAGGCAAGGT
     AACTTGAGAC ACGCTTCTTA AACACACACC TGTAAACTCT CCTGTCAAAC CTCCGTTCCA

901  ATTTTAGTAG ATTTAAAGAA TTTGAATCTT GTTTGCAAGT TGGGGCATAT ACTGAGAAAG
     TAAAATCATC TAAATTTCTT AAACTTAGAA CAAACGTTCA ACCCCGTATA TGACTCTTTC

961  AGAAGACAAT GCAGATAAAT TGATATATTT ATTATGATGT ATGTTCAATA TGAAAGATCA
     TCTTCTGTTA CGTCTATTTA ACTATATAAA TAATACTACA TACAAGTTAT ACTTTCTAGT

1021 CAAAATATAA CATACATNNA TCTTACTTAA CATACCTCAG TTTTAGAGCT ACCGTATGTA
     GTTTTATATT GTATGTANNT AGAATGAATT GTATGGAGTC AAAATCTCGA TGGCATACAT

1081 GAAGAGTCCA TTTCTATTTA GGTAAGTTCC TTTAGTCCTT TTATTACTGG GCACTCTTAA
     CTTCTCAGGT AAAGATAAAT CCATTCAAGG AAATCAGGAA AATAATGACC CGTGAGAATT

1141 TTACATGTAG CTTGAAATAT GTCCAGTTTG AGCAGTGAAC TGAAAATGTC ATGTGATTAA
     AATGTACATC GAACTTTATA CAGGTCAAAC TCGTCACTTG ACTTTTACAG TACACTAATT

1201 GTACATATAT AATTTTTTTT CATAGTAGGT CAATAACCTC CTTTTATTGA CTAATGAATC
     CATGTATATA TTAAAAAAAA GTATCATCCA GTTATTGGAG GAAAATAACT GATTACTTAG

1261 ACTTCTCTAA TGATTATACG
     TCAAGAGATT ACTAATATGC
```

FIG. 75A

```
           10         20         30         40         50         60
            |          |          |          |          |          |
  1 AATCAAAATA AAACAGTTAA AGTTTGATTA CTATAATCAA ACACAAAAAA AATGAATATT
    TTAGTTTTAT TTTGTCAATT TCAAACTAAT GATATTAGTT TGTGTTTTTT TTACTTATAA

 61 ATCTTTTATG TCAGTAGAGG GTGAATGAAT CCTTCAGGAT TTTGATGATA GTATCAGATA
    TAGAAAATAC AGTCATCTCC CACTTACTTA GGAAGTCCTA AAACTACTAT CATAGTCTAT

121 CCCAGCACTA TGCTAGAAGT TGTGAAGAAT TCACGAGATG AATAAATCAC AGATTCTGTC
    GGGTCGTGAT ACGATCTTCA ACACTTCTTA AGTGCTCTAC TTATTTAGTG TCTAAGACAG

181 CTCAAAATGG TTAGATCTAT TCAGGAAACA AAGCTAAAAA AACCCCACCA ATAACTAAAA
    GAGTTTTACC AATCTAGATA AGTCCTTTGT TTCGATTTTT TTGGGGTGGT TATTGATTTT

241 ATCAACCAAA TGAAAAACAA CAATCATAAA ATAAGTAAGT ACCTATAGAA AGAAAAGCTC
    TAGTTGGTTT ACTTTTTGTT GTTAGTATTT TATTCATTCA TGGATATCTT TCTTTTCGAG

301 AGAGGAGGTA AAAAGATAAC TCTTCCAAAA GGAATACTAT ATACTGTAAA CTGTGTACTG
    TCTCCTCCAT TTTTCTATTG AGAAGGTTTT CCTTATGATA TATGACATTT GACACATGAC

361 ATAGAAGGAA GAATTAGAAA NNNNNNNNTG TAAGTGGCAT ACATACTAAG CTAGTGTGAA
    TATCTTCCTT CTTAATCTTT NNNNNNNNAC ATTCACCGTA TGTATGATTC GATCACACTT
```

FIG. 75B

```
421 CACAAGCCTA AATATGTAGT TGCTTCACAG AAGGTTAGAA GTAAATTAAC CTCATGAATT
    GTGTTCGGAT TTATACATCA ACGAAGTGTC TTCCAATCTT CATTTAATTG GAGTACTTAA

481 TCTTGAGAGA ACTTGTAAGG ACTAAGCTTT CGATTTTGGA GAAAGATTTT AATACCAAAT
    AGAACTCTCT TGAACATTCC TGATTCGAAA GCTAAAACCT CTTTCTAAAA TTATGGTTTA

541 AAAAAGTACC TTTGTTTGGT AATCTCAATC ATTATAATAG TGCTTAGATA ATACCTAGGA
    TTTTTCATGG AAACAAACCA TTAGAGTTAG TAATATTATC ACGAATCTAT TATGGATCCT

601 ACAAATTAAA TATTAAATTT ACTTTAAAAA AAAGTACATG ATTGGGGAAT CACAACTGGC
    TGTTTAATTT ATAATTTAAA TGAAATTTTT TTTCATGTAC TAACCCCTTA GTGTTGACCG

661 CTTACTAGAT TCTCTNNNNN NATATGCACT GAAAAGAATG AAAAACACTG AACCAAATAT
    GAATGATCTA AGAGANNNNN NTATACGTGA CTTTTCTTAC TTTTTGTGAC TTGGTTTATA

721 NTGTTTTTTT AAGTTTAAAA TTAAATTGGA AAAAAATAGT AAGGAATATC AGAAGCAAAA
    NACAAAAAAA TTCAAATTTT AATTTAACCT TTTTTTATCA TTCCTTATAG TCTTCGTTTT
```

FIG. 75C

```
781  AAATAAAATG AAAGCAAGAA TCCTCAGAGG TAGCACGAAA TTTGGCTTTG CTTAGATGGA
     TTTATTTTAC TTTCGTTCTT AGGAGTCTCC ATCGTGCTTT AAACCGAAAC GAATCTACCT

841  TCTATCAAAG CTATGGCCCA TGAAAAGGAT TCAGGAGTTA GTTTAAAGCT GGTTCACATA
     AGATAGTTTC GATACCGGGT ACTTTTCCTA AGTCCTCAAT CAAATTTCGA CCAAGTGTAT

901  ATGGAATCTA GCAGAACACT GTGCATAAAG GTGGTCTAAG AACAACAATA TCCTGACCAG
     TACCTTAGAT CGTCTTGTGA CACGTATTTC CACCAGATTC TTGTTGTTAT AGGACTGGTC

961  GTGAGGGGGC TCACNCTNAA TNCCAGCACT TTGGGAGCCC AAGGTGGGTG GATCACGAGG
     CACTCCCCCG AGTGNGANTT ANGGTCGTGA AACCCTCGGG TTCCACCCAC CTAGTGCTCC

1021 TCAGGAGTTT GAGACCAGCC TGACCAACAT GGTGAAACCG CGTCTCTACT AAAAATAGAA
     AGTCCTCAAA CTCTGGTCGG ACTGGTTGTA CCACTTTGGC GCAGAGATGA TTTTTATCTT

1081 AAATTAGCCG NGCCTACGTG CTTCTAATCC CAGCTGAACT CAGGAGACTG AGACAGGAGA
     TTTAATCGGC NCGGATGCAC GAAGATTAGG GTCGACTTGA GTCCTCTGAC TCTGTCCTCT

1141 ATCACTTGAA CCCAGCATGC AAGCTTNNNN NNGCCACTGC ACTCCAGCCT AGGGTGCAAA
     TAGTGAACTT GGGTCGTACG TTCGAANNNN NNCGGTGACG TGAGGTCGGA TCCCACGTTT

1201 AAAAAAAAAA ANGACACATT ACTCAGGTAA GGTAATCAAT AA
     TTTTTTTTTT TNCTGTGTAA TGAGTCCATT CCATTAGTTA TT
```

FIG. 76A

```
- AAGGTAAAAATTATCTCTTTTTTTCTCTCCCCCAATGTAAAAAGTTATAG -
  ||||||||||||||||||||||||||||||||||||||||||||||||||
- AAGGTAAAAATTATCTCTTTTTTTCTCTCCCCCAATGTAAAAAGTTATAG -

- TGGGTTTTACATGTGTAGAATCATTTTCTTAAAACTTTATGAATACCATT -
  ||||||||||||||||||||||||||||||||||||||||||||||||||
- TGGGTTTTACATGTGTAGAATCATTTTCTTAAAACTTTATGAATACCATT -

- ATTTTCTTGTATTCTGTGACATGCCCACCTTACAGAGAGGACACATTTAC -
  ||||||||||||||||||||||||||||||||||||||||||||||||||
- ATTTTCTTGTATTCTGTGACATGCCCACCTTACAGAGAGGACACATTTAC -

- TAGGTTATATCCCGGGGTTAAATTCGAGCATTGGAATTTGGCCAGTGTAG -
  ||||||||||||||||||||||||||||||||||||||||||||||||||
- TAGGTTATATCCCGGGGTTAAATTCGAGCATTGGAATTTGGCCAGTGTAG -

- ATGTTTAGAGTGAACAGAACAAATTTTTCTGTGCTTACAGGTTATGGCTG -
  ||||||||||||||||||||||||||||||||||||||||||||||||||
- ATGTTTAGAGTGAACAGAACAAATTTTTCTGTGCTTACAGGTTATGGCTG -

- TGGCCTACAAGAAGCATGCACTGGGTTTATTATTAACTTTCAGTATCTTT -
  ||||||||||||||||||||||||||||||||||||||||||||||||||
- TGGCCTACAAGAAGCATGCACTGGGTTTATTATTAACTTTCAGTATCTTT -

- GTTTTAAATATTTTCTACAAAAATGTTTACTAAATTAAATTGTAGTATGA -
  ||||||||||||||||||||||||||||||||||||||||||||||||||
- GTTTTAAATATTTTCTACAAAAATGTTTACTAAATTAAATTGTAGTATGA -

- ATTGTTATAAATAATGAGGGAAAACAATTTACACATAGCAAATTTAAAAA -
  ||||||||||||||||||||||||||||||||||||||||||||||||||
- ATTGTTATAAATAATGAGGGAAAACAATTTACACATAGCAAATTTAAAAA -

- TTACTGTCATTTGATTTGTTAATATATTTTTCTCTTTAGTGGGAAATTAA -
  ||||||||||||||||||||||||||||||||||||||||||||||||||
- TTACTGTCATTTGATTTGTTAATATATTTTTCTCTTTAGTGGGAAATTAA -

- ATTTTAAAAAATTCCCTTTCGACTGTAGAACAAATAGGAATTTGGCCTGT -
```

FIG. 76B

```
  ||||||||||||||||||||||||||||||||||||||||||||||||||
- ATTTTAAAAAATTCCCTTTCGACTGTAGAACAAATAGGAATTTGGCCTGT -

- GGGGTCTACTTGCTTATTATATTTGTAAGCTAGTGGTAGGAAATAGCAAA -
  ||||||||||||||||||||||||||||||||||||||||||||||||||
- GGGGTCTACTTGCTTATTATATTTGTAAGCTAGTGGTAGGAAATAGCAAA -

- TGCTCACTACCACTAATAAGAACATTTCTAAATCTGATGTTCTGAGGATT -
  ||||||||||||||||||||||||||||||||||||||||||||||||||
- TGCTCACTACCACTAATAAGAACATTTCTAAATCTGATGTTCTGAGGATT -

- TTTAGAGCTTATAGTAGCAAAAAGAAAAGGGAAATTCTATCCGAGATGTC -
  ||||||||||||||||||||||||||||||||||||||||||||||||||
- TTTAGAGCTTATAGTAGCAAAAAGAAAAGGGAAATTCTATCCGAGATGTC -

- CTTTGTTGTAGGCCTAATGAGAAAAGGTTGAAGATAAAGTTCTGGTACTC -
  ||||||||||||||||||||||||||||||||||||||||||||||||||
- CTTTGTTGTAGGCCTAATGAGAAAAGGTTGAAGATAAAGTTCTGGTACTC -

- ATTTAAGTGTAATATTGAAAATTGATATTACCGAATCTGGAACAACCAAT -
  ||||||||||||||||||||||||||||||||||||||||||||||||||
- ATTTAAGTGTAATATTGAAAATTGATATTACCGAATCTGGAACAACCAAT -

- TTAAAATAAGGAAAGAAAGACACTGTGTTTTCT -
  |||||||||||||||||||||||||||||||||
- TTAAAATAAGGAAAGAAAGACACTGTGTTTTCT -
```

FIG. 77A

```
             10         20         30         40         50         60
              |          |          |          |          |          |
  1 AGAAAACACA GTGTCTTTCT TTCCTTATTT TAAATTGGTT GTTCCAGATT CGGTAATATC
    TCTTTTGTGT CACAGAAAGA AAGGAATAAA ATTTAACCAA CAAGGTCTAA GCCATTATAG

 61 AATTTTCAAT ATTACACTTA AATGAGTACC AGAACTTTAT CTTCAACCTT TTCTCATTAG
    TTAAAAGTTA TAATGTGAAT TTACTCATGG TCTTGAAATA GAAGTTGGAA AAGAGTAATC

121 GCCTACAACA AAGGACATCT CGGATAGAAT TTCCCTTTTC TTTTTGCTAC TATAAGCTCT
    CGGATGTTGT TTCCTGTAGA GCCTATCTTA AAGGGAAAAG AAAAACGATG ATATTCGAGA

181 AAAAATCCTC AGAACATCAG ATTTAGAAAT GTTCTTATTA GTGGTAGTGA GCATTTGCTA
    TTTTTAGGAG TCTTGTAGTC TAAATCTTTA CAAGAATAAT CACCATCACT CGTAAACGAT

241 TTTCCTACCA CTAGCTTACA AATATAATAA GCAAGTAGAC CCCACAGGCC AAATTCCTAT
    AAAGGATGGT GATCGAATGT TTATATTATT CGTTCATCTG GGGTGTCCGG TTTAAGGATA

301 TTGTTCTACA GTCGAAAGGG AATTTTTTAA AATTTAATTT CCCACTAAAG AGAAAAATAT
    AACAAGATGT CAGCTTTCCC TTAAAAAATT TTAAATTAAA GGGTGATTTC TCTTTTTATA

361 ATTAACAAAT CAAATGACAG TAATTTTTAA ATTTGCTATG TGTAAATTGT TTTCCCTCAT
    TAATTGTTTA GTTTACTGTC ATTAAAAATT TAAACGATAC ACATTTAACA AAAGGGAGTA

421 TATTTATAAC AATTCATACT ACAATTTAAT TTAGTAAACA TTTTTGTAGA AAATATTTAA
    ATAAATATTG TTAAGTATGA TGTTAAATTA AATCATTTGT AAAAACATCT TTTATAAATT
```

FIG. 77B

```
481 AACAAAGATA CTGAAAGTTA ATATNAAACC CAGTGCATGC TTCTTGTAGG CCACAGCCAT
    TTGTTTCTAT GACTTTCAAT TATANTTTGG GTCACGTACG AAGAACATCC GGTGTCGGTA

541 AACCTGTAAG CACAGAAAAA TTTGTTCTGT TACTCTAAAC ATCTACACTG GCCAAATTCC
    TTGGACATTC GTGTCTTTTT AAACAAGACA ATGAGATTTG TAGATGTGAC CGGTTTAAGG

601 AATGCTCGAA TTTAACCCCG GGATATAACC TAGTAAATGT GTCCTCTCTG TAAGGTGGGC
    TTACGAGCTT AAATTGGGGC CCTATATTGG ATCATTTACA CAGGAGAGAC ATTCCACCCG

661 ATGTCACAGA ATACAAGAAA ATAATGGTAT TCATAAAGTT TTAAGAAAAT GATTCTACAC
    TACAGTGTCT TATGTTCTTT TATTACCATA AGTATTTCAA AATTCTTTTA CTAAGATGTG

721 ATGTAAAACC CACTATAACT TTTTACATTG GGGGAGAGAA AAAAAGAGAT AATTTTTACC
    TACATTTTGG GTGATATTGA AAAATGTAAC CCCCTCTCTT TTTTTCTCTA TTAAAAATGG

781 TT
    AA
```

FIG. 78A

```
            10         20         30         40         50         60
             |          |          |          |          |          |
  1 GATGCTATTT GGGCAATTTC TTATTGACAG TTTTGAAATG TTAGGCTTTT ATCTCCATTT
    CTACGATAAA CCCGTTAAAG AATAACTGTC AAAACTTTAC AATCCGAAAA TAGAGGTAAA

 61 TTTAGTACTT AAATTTTCCA ACATGGGTGT TGCTTGTTAT TTTATCAGTA TAAAATAGAA
    AAATCATGAA TTTAAAAGGT TGTACCCACA ACGAACAATA AAATAGTCAT ATTTTATCTT

121 GAGTGGTTCT GTTCTGGAAT TTAGTATATA CATGAGTATC TAGTGTATGT CAGCCATGAA
    CTCACCAAGA CAAGACCTTA AATCATATAT GTACTCATAG ATCACATACA GTCGGTACTT

181 AATGAACCTT TCAGATGTTT AACTTCAGGG AACCTAATTG AGTCATTGCT CCAGACATTG
    TTACTTGGAA AGTCTACAAA TTGAAGTCCC TTGGATTAAC TCAGTAACGA GGTCTGTAAC

241 TTGCTTTGAA CCCACTATAT TNNNNNNNCT CGGGCAATGA CTCAGTGTGG CAAGGATACT
    AACGAAACTT GGGTGATATA ANNNNNNNGA GCCCGTTACT GAGTCACACC GTTCCTATGA

301 ACTGCAGGCC TGTTTCTGGA AGGCACTGGA CTCCTCTGAT GCAAACTTTG GCCAGGGACT
    TGACGTCCGG ACAAAGACCT TCCGTGACCT GAGGAGACTA CGTTTGAAAC CGGTCCCTGA

361 CCTTGATAGC TCTTAAATAG ATGCTGCACC AACACTCTCT TTCTTTTCTC TCTTTTTCTT
    GGAACTATCG AGAATTTATC TACGACGTGG TTGTGAGAGA AAGAAAAGAG AGAAAAAGAA
```

FIG. 78B

```
421 TATTCAATAT TAGACTACAA GCAGTCTAAG GACTTCTCAG GGTTTCTAGC TCTCTCTCAT
    ATAAGTTATA ATCTGATGTT CGTCAGATTC CTGAAGAGTC CCAAAGATCG AGAGAGAGTA

481 TTCACACATG CTTTCCTAGT AATCTCTACT CAIATATCTT ACTGCTACGC TGGGGCCAGA
    AAGTGTGTAC GAAAGGATCA TTAGAGATGA GTATATAGAA TGACGATGCG ACCCCGGTCT

541 TAACNNNNNN CTTCCATTTT GTTTTTATCT CTATTCTTCT TCCCCTTCTG CTTTCATTAT
    ATTGNNNNNN GAAGGTAAAA CAAAAATAGA GATAAGAAGA AGGGGAAGAC GAAAGTAATA

601 TGAAACTTTC TGCTTTCATT ATTGAAACTT TCCCAGATTT GTTCTGCTTA ACCTGGCATT
    ACTTTGAAAG ACGAAAGTAA TAACTTTGAA AGGGTCTAAA CAAGACGAAT TGGACCGTAA

661 GGAACTGTTT CCTCTTCCCT GTGCTGCTTT CTCCCATTGC CATGTCCTTT TTTTTTTTTT
    CCTTGACAAA GGAGAAGGGA CACGACGAAA GAGGGTAACG GTACAGGAAA AAAAAAAAAA

721 TTTTTTTTTT TGAGACAGTG TCACTCTGTT GCCCAGGCTG GAGTGCAATG GTGCAATCTT
    AAAAAAAAAA ACTCTGTCAC AGTGAGACAA CGGGTCCGAC CTCACGTTAC CACGTTAGAA
```

FIG. 78C

```
781  GGCCACTGCA ACCCCCGCCT CCCGGGTTCA AGTGATTCTC CTGCCTCAGC CTCCTGAGTA
     CCGGTGACGT TGGGGGCGGA GGGCCCAAGT TCACTAAGAG GACGGAGTCG GAGGACTCAT

841  GCTGGGATTA CAGGTGCCCA CCACTATGCC CGGCTGATTT TTGTATTTTT AGTAGAGATN
     CGACCCTAAT GTCCACGGGT GGTGATACGG GCCGACTAAA AACATAAAAA TCATCTCTAN

901  NNNNNNNTTT CACCATNGCT GATCAGGCTG GTCTCGAACT CCTGACCGCA GTGANTCCGC
     NNNNNNNAAA GTGGTANCGA CTAGTCCGAC CAGAGCTTGA GGACTGGCGT CACTNAGGCG

961  CCTCCTTGGC CTCCCAAAGT GCTGACATTA CAGGCATGAG TCACTGCGNC CAGCCACCAT
     GGAGGAACCG GAGGGTTTCA CGACTCTAAT GTCCGTACTC AGTGACGCNG GTCGGTGGTA

1021 TATTCTCTAG AGGTGAGAGA ACACTGGCTC TTCTAACAAG TTGAAATTTG ATAGAGACC
     ATAAGAGATC TCCACTCTCT TGTGACCGAG AAGATTGTTC AACTTTAAAC TATCTCTGG
```

FIG. 79A

```
             10         20         30         40         50         60
              |          |          |          |          |          |
  1 CACAAAAAAA GATTATTAGC CACAAAAAAA CCTTGAAGTA ACGCATTAAA ATGTTAATGG
    GTGTTTTTTT CTAATAATCG GTGTTTTTTT GGAACTTCAT TGCGTAATTT TACAATTACC

 61 ATTCACTTTA TTGAGCATCT GCTCATAATA CTTTAATGAG TGCAAAGTGC TTTGAATATA
    TAAGTGAAAT AACTCGTAGA CGAGTATTAT GAAATTACTC ACGTTTCACG AAACTTATAT

121 ATACGTCATT TAAACCTTAC CATAATTCTG AGGAATTGCT ACCTCCACTT CACAGATGGG
    TATGCAGTAA ATTTGGAATG GTATTAAGAC TCCTTAACGA TGGAGGTGAA GTGTCTACCC

181 GCACAGGAGG CTTAGATAAC ATGCCCAAAG TCATGCTTCT AGTAAATGGA TATAATTAAG
    CGTGTCCTCC GAATCTATTG TACGGGTTTC AGTACGAAGA TCATTTACCT ATATTAATTC

241 ATTCAAATTA TTGATAAGAA TTTGATCTGC CTTACCAGTA TCTAGTAGTA AATCTAAAAG
    TAAGTTTAAT AACTATTCTT AAACTAGACG GAATGGTCAT AGATCATCAT TTAGATTTTC

301 CGCTTTCCAG AGCATGTGCT GTTGATAGAG CTTGATGTCT AACTCTCTGA AATTTTCCAT
    GCGAAAGGTC TCGTACACGA CAACTATCTC GAACTACAGA TTGAGAGACT TTAAAAGGTA

361 TCTTATTTGT CTCACTGGTA TATAGTTATT TTTTACTACT TTCATACACC TACTAAGAAG
    AGAATAAACA GAGTGACCAT ATATCAATAA AAAATGATGA AAGTATGTGG ATGATTCTTC
```

FIG. 79B

```
421 ACAGGAGGAT CAAAGATAGG ATTTCATTTA GAATGCCTAA AGCTTCACGT ATTTTAATTC
    TGTCCTCCTA GTTTCTATCC TAAAGTAAAT CTTACGGATT TCGAAGTGCA TAAAATTAAG

481 AGAATAAGAT TCAGGCAGAC CACCAGTATA TGCCATGGTC CCTGGTTATC TTTCAGCAGG
    TCTTATTCTA AGTCCGTCTG GTGGTCATAT ACGGTACCAG GGACCAATAG AAAGTCGTCC

541 TGACCGAGAA AGAAAACATG GTAATGTTTA TGAAATGGTG GGTTCTTGTA GTTTCACTTC
    ACTGGCTCTT TCTTTTGTAC CATTACAAAT ACTTTACCAC CCAAGAACAT CAAAGTGAAG

601 AACATATCTG CCTTTACTGT ATTAAGATGA TGGATTAACT TATTCTTGAT ATGGGCATGT
    TTGTATAGAC GGAAATGACA TAATTCTACT ACCTAATTGA ATAAGAACTA TACCCGTACA

661 AAAACAATAT ACTTTTACTA AACAGCTACA GAGAGACAAA TGTGTTTCCA GACAAACTTA
    TTTTGTTATA TGAAAATGAT TTGTCGATGT CTCTCTGTTT ACACAAAGGT CTGTTTGAAT

721 AGAGACTGAG TGTTCAAACT GAATAATCTC GACCTTAATT GTAACTATAT TTTATGAAAT
    TCTCTGACTC ACAAGTTTGA CTTATTAGAG CTGGAATTAA CATTGATATA AAATACTTTA
```

FIG. 79C

```
781  CCAGCTGTAA GGCAAAAACA GACTTCTTTG GGCCTACCAC GGGCATTTTG TTCCTGTTAN
     GGTCGACATT CCGTTTTTGT CTGAAGAAAC CCGGATGGTG CCCGTAAAAC AAGGACAATN

841  NNNTACTCCA AACCTTAAAC CCACGTCCAC TTAAATAATG GCCTGGAAAT AAATGTCATT
     NNNATGAGGT TTGGAATTTG GGTGCAGGTG AATTTATTAC CGGACCTTTA TTTACAGTAA

901  ATCTGATATT ATACTGAGAT GTTTAGTTAT GAAATCAAAA GTGGAGAATT TCAATCTGTC
     TAGACTATAA TATGACTCTA CAAATCAATA CTTTAGTTTT CACCTCTTAA AGTTAGACAG

961  CTGTAAGCTT TCTCTGCGGT CACGACCCTC ATGCACTCAG GCTGTGCGGT GCAGCATGCT
     GACATTCGAA AGAGACGCCA GTGCTGGGAG TACGTGAGTC CGACACGCCA CGTCGTACGA

1021 CTGTCATGTC TGTTTTCTTC TGCCTGTACA CGGGTGGTTG TTCCTGTCTA CCTGTTTGAG
     GACAGTACAG ACAAAAGAAG ACGGACATGT GCCCACCAAC AAGGACAGAT GGACAAACTC

1081 GAAATATGAA TACGTNNNNN NCTAGAATCT ACTGCACATG CAATAAGGAA ACAATCAGTA
     CTTTATACTT ATGCANNNNN NGATCTTAGA TGACGTGTAC GTTATTCCTT TGTTAGTCAT

1141 AGAATCACTT TCTCGTGGAA AATTCATTAG AATTAACATC TCGTTTTAAA ATGCTCTATC
     TCTTAGTGAA AGAGCACCTT TTAAGTAATC TTAATTGTAG AGCAAAATTT TACGAGATAG
```

FIG. 79D

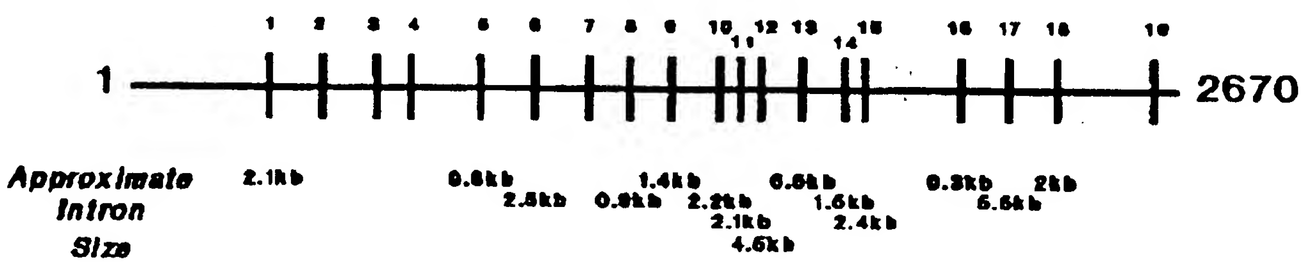
```
1201 AAAGTGTAAA TAATTCCTCT CTCTTTTCCC TTTTTCACTA AGGAGTTTGT ATATTAAACA
     TTTCACATTT ATTAAGGAGA GAGAAAAGGG AAAAAGTGAT TCCTCAAACA TATAATTTGT

1261 GAATTTCAAG TAATGTATTA TAAATTTATT TAANNTATTT ACAATAAAAT GCCACGTATA
     CTTAAAGTTC ATTACATAAT ATTTAAATAA ATTNNATAAA TGTTATTTTA CGGTGCATAT

1321 AGCATCAAGC AACATGANNN NNNCATTGGT AGAAAGCACA ATACATAGTC AAAACAGCAG
     TCGTAGTTCG TTGTACTNNN NNNGTAACCA TCTTTCGTGT TATGTATCAG TTTTGTCGTC

1381 AGTATTAAAT AAACAGAAAA TTTGCAAAAG GCAAGTAAAG AATATACATA TACTTAATTA
     TCATAATTTA TTTGTCTTTT AAACGTTTTC CGTTCATTTC TTATATGTAT ATGAATTAAT

1441 TACATAAAAT ATTGATACAG GAGGTAGAAA GAAATTTAGT AAGCAGATAA TGGGGGCAAC
     ATGTATTTTA TAACTATGTC CTCCATCTTT CTTTAAATCA TTCGTCTATT ACCCCCGTTG

1501 AGAGTCCTCA GCAGAGCTTC CCTTCTAACA AAAAGCAGCC CAATAAATTA TTTTTTTTTT
     TCTCAGGAGT CGTCTCGAAG GGAAGATTGT TTTTCGTCGG GTTATTTAAT AAAAAAAAAA

1561 CTAACAAAAA GCAGCCTGAA AAATCGAGCT GCAAACATAG ATTAGCAATC GGCTGAAAGT
```

FIG. 79E

```
     GATTGTTTTT CGTCGGACTT TTTAGCTCGA CGTTTGTATC TAATCGTTAG CCGACTTTCA

1621 GCGGGAGAAT GCTGGCAGCT GTGCCAATAG TAAAGGGCTA CCTGGAGCCG GGCGCGTGGC
     CGCCCTCTTA CGACCGTCGA CACGGTTATC ATTTCCCGAT GGACCTCGGC CCGCGCACCG

1681 TCACGCTGTA ATCCCAGCAC TTTGGGAGGG CGAGGCAACG CGGATCACCT GAGGTCGGGA
     AGTGCGACAT TAGGGTCGTG AAACCCTCCC GCTCCGTTGC GCCTAGTGGA CTCCAGCCCT

1741 GTTTGAGATC AGCCCGACCA ACATGGAGAA ACCCCGTCTC TACTAAAAAA AAAAAAAAAA
     CAAACTCTAG TCGGGCTGGT TGTACCTCTT TGGGGCAGAG ATGATTTTTT TTTTTTTTTT

1801 AAAGGCAAAA AATGAGCCGG GCATGGTGGC ACATGCCTTG CACATCCCAG CTGAGGCAGG
     TTTCCGTTTT TTACTCGGCC CGTACCACCG TGTACGGAAC GTGTAGGGTC GACTCCGTCC

1861 AGAATTCACT TGAACCTGGG AGGTAGAGAT TGCGGTGAAG CGAGATCACG TCATTGCACT
     TCTTAAGTGA ACTTGGACCC TCCATCTCTA ACGCCACTTC GCTCTAGTGC AGTAACGTGA

1921 CCAGCCTGGG CAAAAAGAGC AAAACTTAGT CTCAAAAAAA AAAANNCAAA GAAAAAA
     GGTCGGACCC GTTTTTCTCG TTTTGAATCA GAGTTTTTTT TTTTNNGTTT CTTTTTT
```

FIG. 80

Genomic Organization of PSM Gene



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/02424

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/12, 15/64; C12Q 1/68; C07K 14/435
US CL :536/23.5; 435/6, 7.1, 320.1, 252.3, 69.3; 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.5; 435/6, 7.1, 320.1, 252.3, 69.3; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

INPADOC, CA
search terms: prostate specific membrane antigen

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A, 94/09820 (SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH) 11 May 1994, see entire document.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date		
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search 29 APRIL 1996	Date of mailing of the international search report 14 MAY 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ANTHONY C. CAPUTA Telephone No. (703) 308-0196